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Genetic Structure of Scent Profiles in Antirrhinum. Implications for the Evolution and Interaction with Pests and Pollinators

Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario

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PhD Thesis

Genetic structure of scent profiles in *Antirrhinum*. Implications for the evolution and interaction with pests and pollinators

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Que la referida Tesis Doctoral, ha sido realizada por Dª. Mª Victoria Ruiz Hernández, dentro del Programa de Doctorado TÉCNICAS AVANZADAS EN INVESTIGACIÓN Y DESARROLLO AGRARIO Y ALIMENTARIO, dando mi conformidad para que sea presentada ante el Comité de Dirección de la Escuela Internacional de Doctorado para ser autorizado su depósito.

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Preface

This document has been done according to the *Universidad Politécnica de Cartagena* regulations. This PhD thesis is formed by different sections including: a general introduction, five chapters which correspond to accepted or under review publications, the conclusions obtained from this work and a section about conference communications. The introduction of this dissertation does not include a “materials and methods” part as they are described within each chapter. Moreover, the bibliographies used in each section are included within each of them. Some references are repeated in the bibliographies of one to several sections.

The present PhD thesis has been financed by the *Ministerio de Educación Cultura y Deporte*, within the *Programa Estatal de Promoción del Talento y su Empleabilidad* and the *Subprograma de Formación de Profesorado Universitario* (FPU) FPU13/03606. Additionally, during the development of the thesis a research stay was performed at the University of Cambridge, in the research group of Dr. Beverley Glover. This research stay was financed by the *Programa de Formación del Personal Docente e Investigador a través de la Movilidad* of the *Universidad Politécnica de Cartagena* (PMPDI UPCT).

Publications derived from the PhD thesis

Weiss, J., Mühlemann, J. K., **Ruiz-Hernández, V.**, Dudareva, N., & Egea Cortines, M. (2016). Phenotypic space and variation of floral scent profiles during late flower development in *Antirrhinum*. Frontiers in Plant Science, 7, 1903. doi:10.3389/fpls.2016.01903

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Ruiz-Hernández, V., Hermans, B., Weiss, J., & Egea Cortines, M. (2017). Genetic analysis of natural variation in *Antirrhinum* scent profiles identifies BENZOIC ACID CARBOXYMETHYL TRANSFERASE as the major locus controlling methyl benzoate synthesis. Frontiers in Plant Science, 8 (27), 13. doi:10.3389/fpls.2017.00027

Impact factor: 3.678 (2017)

Ruiz-Hernández, V., Roca, M. J., Egea Cortines, M., & Weiss, J. (2018). A comparison of semi quantitative methods suitable for establishing volatile profiles. Plant Methods, 14 (1), 67. doi:10.1186/s13007 018 0335 2

Impact factor: 4.269 (2017)

Forthcoming publications derived from the PhD thesis

Ruiz-Hernández, V., Pérez Sanz, F., Arce Gallego, S., Weiss, J., Navarro, P., & Egea Cortines, M. gcProfileMakeR: an R package for automatic identification of constitutive and non constitutive metabolites.

Ruiz-Hernández, V., Joubert, L., Rodríguez Gómez, A., Pattrick, J., Weiss, J., Bielza, P., Glover, B.J., & Egea Cortines, M. Behavioural responses of pest and pollinator to *Antirrhinum* floral scent profiles.

Publications unrelated to the PhD thesis

Egea Gilabert, C., **Ruiz-Hernández, M. V.**, Parra, M. Á., & Fernández, J. a. (2014). Characterization of purslane (*Portulaca oleracea* L.) accessions: Suitability as ready to eat product. Scientia Horticulturae, 172, 73–81. DOI: 10.1016/j.scienta.2014.03.051

Weiss, J., Martos Fuentes, M., Letourneux, L., Terry, M. I., **Ruiz-Hernández, V.**, Fernández, J. A., & Egea Cortines, M. (2018). Diel pattern of circadian clock and storage protein gene expression during seed filling in cowpea (*Vigna unguiculata*). BMC Plant Biology (1), 33. DOI: 10.1186/s12870 018 1244 2

Abstract - Resumen

Abstract

Scents are distinctive phenotypic traits which can be used to characterize species. The interaction between plants and other kingdoms is partly mediated by combinations of Volatile Organic Compounds (VOCs). Floral scents play an important role in pollination and species survival. VOCs emitted from plants can be categorized into two groups: compounds which are emitted constitutively and form the core volatilome of the plant, organ or tissue and compounds that are non-constitutive and result from the induction of certain routes in the secondary metabolism. In the non-targeted analysis of volatile metabolomes important amounts of data are generated and automatization of gas-chromatography mass-spectrometry (GC-MS) outputs is necessary in order to facilitate the analysis of higher amounts of samples. Comparative analysis of scent profiles can be performed by using different semi-quantification methodologies which provide the relative proportions of VOCs.

We characterized the scent emission during flower development of eight *Antirrhinum* wild species: *A. linkianum*, *A. tortuosum*, *A. cirrigherum, A. latifolium, A. meonanthum, A. braun-blanquetii, A. barrelieri*, and *A. graniticum* and two laboratory inbred lines from *A. majus*, 165E and Sippe50. Total scent emission varied during flower development and species. By using scent profiles, in general, species clustered according to the current species phylogeny. Contrastingly, cluster analysis of VOCs did not match synthesis pathways, indicating a relevant role of scent profiles as blends in *Antirrhinum*.

Furthermore, we performed a genetic analysis of *A. linkianum* and *A. majus* (165E) analysing their contrasting emissions of methyl benzoate, methyl cinnamate, acetophenone and ocimene. The study was based on the segregation of these VOCs on an F2 population. Results attributed their emission to one or two *loci* with simple or epistatic interactions. Methyl benzoate was emitted in *A. majus* plants whereas *A. linkianum* did not emit it. Following a candidate gene approach, we found that the *BENZOIC ACID CARBOXYMETHYL TRANSFERASE (BAMT)* from *A. linkianum* was a null allele. Our results indicate that major rearrangements in the promoter region, which include the insertion of an *IDLE MITE* transposon, in *A. linkianum BAMT* cause its lack of emission of methyl benzoate. Differences in scent emission between these two species are mediated by changes in single genes involved in discrete biosynthetic reactions such as the methylation of benzoic acid.

Quantification of VOCs emitted, ideally requires the use of chemical standards and calibration curves. However, VOCs composition is unpredictable and there are not standards for every VOC found. The analysis of scent profiles is more relevant from the point of view of relative abundances of VOCs than absolute quantities. We compared seven different semi-quantitative methods. Furthermore, we explored the usage of nearest components and single calibrators for semi-quantifications. We found that any of the semi-quantification methods based on standards yielded similar or identical statistical results compared to quantification by a true-standard for a compound, except for the method based on standard addition. We discuss the advantages and disadvantages of each method regarding accuracy, experimental variability, acceptance and retrieved quantities.

We developed gcProfileMakeR, an R package that uses common output files from GC-MS as inputs and provides a list of core and non-constitutive volatiles as outputs. By using three functions, the program defines the number and identity of compounds present in each sample and between samples, and finally establishes the thresholds of quality and presence required for grouping VOCs. Metabolites are grouped into three categories, Profile, Non-Constitutive by Frequency and Non-Constitutive by Quality. As a proof of concept, we define the floral volatilomes of *A. majus compacta* (*co*) and *deficiens-nicotianoides* (*def-nic*) mutants. Our results show substantial differences in the composition of VOCs emitted by the flowers.

We tested the preferences of pests and pollinators for distinctive and different floral scent profiles from *A. majus* and *A. linkianum*, and from four recombinant inbred lines (RILs). As pest, we used *Frankliniella occidentalis* whereas as pollinator we used *Bombus terrestris audax*. We established the core volatilome of plant lines by using gcProfileMakeR. We found that bumblebees are attracted by methyl benzoate and thrips are repelled by β-myrcene. Furthermore, we identified compounds that may act as enhancers of attraction/repellence effects on insects when acting in combination with others.

From the work carried out in this PhD dissertation we can draw some general conclusions. Firstly, floral scent profiles are characteristic of *Antirrhinum* species and may be the result of local adaptation to pests and pollinators. Transposons are relevant intermediaries mediating evolution given that they can affect the emission of key volatiles mediating pollinator attraction, such as methyl benzoate. VOCs are important mediators in plant-insect interactions both as key compounds and as blends of scents. The development of new tools that allow the analysis of a higher number of samples and a general agreement on the semi-quantification methods to be used will enhance the reproducibility of data. Breeding programs should take into account results related to the effect of floral VOCs on insect preferences in order to improve the innate defence of crops, yield productions and fruit quality.

Resumen

Los olores son caracteres fenotípicos distintivos que pueden ser utilizados para caracterizar especies. Combinaciones de Compuestos Orgánicos Volátiles (COVs) intervienen en las interacciones entre plantas y otros reinos. Los aromas florales juegan un papel importante en la polinización y la supervivencia de las especies. Los COVs emitidos por las plantas se pueden agrupar en dos clases: compuestos que son emitidos constitutivamente y que forman parte del perfil aromático de la planta, órgano o tejido y compuestos que no se emiten constitutivamente y que son el resultado de la inducción de ciertas rutas del metabolismo secundario. En el análisis no específico de los metabolomas volátiles se generan cantidades considerables de datos. En este sentido, la automatización de los resultados obtenidos por gases masas y espectrometría de masas (GC-MS) es necesaria para facilitar el análisis de un mayor número de muestras. El análisis comparativo de los perfiles aromáticos se puede realizar utilizando distintos métodos de semi-cuantificación que proporcionan información acerca de las proporciones relativas de COVs.

Se caracterizó la emisión de aromas durante el desarrollo de las flores de ocho especies silvestres: *Antirrhinum linkianum*, *A. tortuosum*, *A. cirrigherum, A. latifolium, A. meonanthum, A. braun-blanquetii, A. barrelieri*, y *A. graniticum;* además de dos líneas consanguíneas de *A. majus*, 165E y Sippe50. La emisión total varió entre las especies y durante el desarrollo de las flores. Utilizando los perfiles aromáticos, en general, las especies se agruparon de acuerdo a la actual filogenia. En contraste, el análisis de clústeres de los COVs no se agrupó de acuerdo a las rutas metabólicas, lo que indica el papel relevante de los perfiles aromáticos como mezclas específicas en *Antirrhinum*.

Por otro lado, realizamos un análisis genético de *A. linkianum* y *A. majus* (165E). Analizamos sus emisiones contrastantes de metil benzoato, metil cinamato, acetofenona y ocimeno. El estudio se basó en la segregación de estos volátiles en una población F2. Los resultados indicaron que la emisión de estos compuestos se atribuye a uno o dos *loci* con interacciones simples o epistáticas. Metil benzoato se emite principalmente en plantas de *A. majus* y no en *A. linkianum*. Siguiendo un enfoque de gen candidato, se halló que la *BENZOIC ACID CARBOXYMETHYL TRANSFERASE (BAMT)* de *A. linkianum* era un alelo nulo. Nuestros resultados indican que cambios considerables en la zona promotora, que incluyen la inserción de un transposón *IDLE MITE* en *A. linkianum BAMT,* causan su falta de emisión de metil benzoato. Cambios en genes aislados involucrados en la biosíntesis de volátiles (como la metilación del ácido benzoico) establecen las diferencias en la emisión de aromas entre estas dos especies.

La cuantificación de los COVs emitidos requiere, idealmente, del uso de estándares químicos y de curvas de calibrado. Sin embargo, la composición aromática es impredecible y no hay estándares para cada volátil hallado. En el análisis de perfiles aromáticos es más relevante la abundancia relativa de los compuestos que las abundancias absolutas de los mismos. Se compararon siete métodos semi-cuantitativos diferentes. Además, se estudió el uso de los componentes más cercanos y de calibradores únicos para la semi-cuantificación. Se encontró que cualquiera de los métodos basados en estándares obtuvieron resultados similares o idénticos cuando se comparaba con las cuantificaciones por estándares verdaderos, excepto en

el método de adición estándar. Se discuten las ventajas y desventajas de cada método con respecto a la precisión, variabilidad experimental, aceptación y cantidades obtenidas.

Se desarrolló gcProfileMakeR, un paquete de R que usa archivos obtenidos habitualmente por GC-MS y los utiliza como archivos de entrada para producir una lista de compuestos volátiles constitutivos y no constitutivos como resultado. Utilizando tres funciones, el programa define el número y la identidad de los compuestos presentes en cada muestra y entre las muestras. Finalmente, establece los umbrales de calidad y de presencia requeridos para agrupar los compuestos hallados. Los metabolitos son agrupados en tres categorías: Perfil, No-Constitutivos por Frecuencia y No-Constitutivos por Calidad. Como prueba de concepto, definimos el volatiloma floral de los mutantes de *A. majus compacta* (*co*) y *deficiens-nicotianoides* (*def-nic*). Nuestros resultados muestran diferencias sustanciales en la composición de COVs emitidos por las flores.

Se estudiaron las preferencias de plagas y polinizadores por distintos y distintivos perfiles aromáticos de *A. majus*, *A. linkianum* y cuatro líneas recombinantes consanguíneas (RILs). Como plaga, se utilizó *Frankliniella occidentalis* mientras que como polinizador se utilizó *Bombus terrestris audax*. Se estableció el volatiloma constitutivo de las líneas analizadas con gcProfileMakeR. Se encontró que los abejorros eran atraídos por metil benzoato y los trips son ahuyentados por β-myrceno. Además, se identificaron compuestos que pueden actuar como potenciadores de los efectos atrayentes o repelentes cuando actúan en combinación con otros COVs.

Del trabajo llevado a cabo en esta tesis doctoral se obtienen varias conclusiones generales. Por un lado, los perfiles aromáticos de las flores de *Antirrhinum* son característicos de las especies estudiadas y pueden ser el resultado de adaptaciones locales a plagas y polinizadores. Además, los transposones son intermediarios relevantes que median en la evolución, ya que pueden afectar a la emisión de volátiles clave en la atracción de polinizadores, como metil benzoato. Los COVs son mediadores importantes en las interacciones entre plantas e insectos, tanto como compuestos clave o como mezclas específicas de aromas. El desarrollo de nuevas herramientas que permiten el análisis de un mayor número de muestras y un consenso general de los métodos de semi-cuantificación a utilizar, estimulará la reproducibilidad de los datos obtenidos. Los programas de mejora genética deberían de tener en cuenta los resultados relacionados con el efecto de los volátiles sobre las preferencias de los insectos para mejorar la defensa innata de los cultivos, las producciones y la calidad de los frutos.

Introduction

From genes to metabolites

While the concept of gene is continuously evolving, several seminal steps have brought us to the current model. In a way, our understanding of the gene has evolved with technology development. The original concept of a gene identified as a functional unit subject to mutation and recombination, was largely based on the identification of genes by allelic segregations (Lewis, 1951).

The collinearity between the coding region of a gene and the coded protein is a hypothesis that has played a fundamental role in the concept of the gene. The relationship between genes and enzymes was developed in the early XXth century by Garrod, Durham and Wheldale, working on human sicknesses, melanic pigments in mammals and vegetables (cited in Rose, 1983). The one gene one enzyme hypothesis was developed by works in *Neurospora* and *Escherichia coli* (Beadle and Tatum, 1941; Lederberg and Tatum, 1946) which constituted the basis for the molecular concept of the gene.

A significant advance resulted from using bacteria and viruses as well as mutagenesis. Seminal works by Muller and Auerbach showed the mutagenic effects of radiations and chemical mutagenes which led to further analysis and improvement in the development of the concept of gene (Muller, 1927; Muller, 1928; Auerbach and Robson, 1946). Between 1954 and 1962, Benzer developed the concept of cistron by works carried out in the rII *locus* of *Enterobacteria phage T4* (Beurton et al., 2000). These works showed that genetic units, or genes, have a large number of mutation and recombination possibilities.

The concept of the gene as it stands today, is that of a DNA fragment producing a gene product. Sydney Brenner, proposed in 2000 that a gene refers to any DNA fragment that has been subject to mutation, linkage analysis or any open reading frame, even if there is no current evidence of its translation (Brenner et al., 2000). As the advances in small peptide and small RNA research show, this last point of view is probably correct (Nakayama et al., 2017; Cui et al., 2018b).

These basic and remarkable findings leaded to the basis of current biology, used in any expertise field outside pure genetics studies. DNA is the source of detailed instructions to construct life. Commands encoded in genes result in the synthesis of RNA and proteins. Transcription of DNA into RNA mediates the ultimate synthesis of proteins and their expression. Proteins are synthesized after translation of RNA and, at the same time, they are the bricks that support life in cells and the machinery that mediates in the expression and replication of DNA (Nobelprize.org).

Cell metabolism is involved in the conversion of substrates to energy to support cellular processes, the conversion of energy to create new molecules and the elimination of cell waste. Metabolism is classified into primary and secondary. Primary metabolism is involved in the synthesis and degradation of the major building blocks in biological systems. Thus, it includes the basic process of photosynthesis in plants, synthesis of amino acids, lipids, carbohydrates and the nucleotides and deoxynucleotides required for RNA and DNA synthesis. The secondary metabolism is comprised by specific molecules that may play important biological functions but may not be absolutely required for survival. Metabolites are intermediate and end products of metabolism. The functions of metabolites are varied and can be classified into the level of cell functioning they are involved in.

Currently the advent of genetic analysis of traits has allowed the understanding of complex systems, including the different points of decision required for a given process within cell metabolism. Examples include the disentangling of the cell cycle (Nurse, 2008), embryonic development (Nusslein Volhard and Wieschaus, 1980) or the identification of dsRNAs as post transcriptional gene silencing coordinators (Fire et al., 1998).

Genomes evolution and coding potential

Genomes hold the complete set of genetic information and contain all the instructions necessary for the functioning of organisms. The coding potential of genomes defines the adaptation of organisms to their environment and the hereditability of characters. While the macroscopic changes seen between species are the result of macroevolution, the current hypothesis is that the driving force underneath the phenotypic changes is based on micro evolutionary changes which occur at the DNA level (Irish and Litt, 2005).

Microevolution changes may or may not translate into visible phenotypes, and include mutation, genome duplication and recombination. The resulting forces on DNA and genes include stochastic effects such as mutation, recombination and genetic drift, and systematic effects such as evolutionary pressure and migration.

In the kingdom *Plantae* the sizes of genomes vary broadly, from 135 MB in *Arabidopsis* to 40 GB in *Pinus* (Morse et al., 2009). The high variation found in the size of plant genomes is related to the activity of transposable elements. Furthermore, all plant genomes have gone through multiple and cyclical episodes of duplication followed by manifold fragmentary processes (Wendel et al., 2016). Genome duplication processes result in the doubling of genes which has contributed to the evolution of species by the diversification of paralogs (Irish and Litt, 2005).

Sequence homology and synteny analysis have provided in the last decades an outstanding broadening of science frontiers, as we are able to identify the evolution and structure of plant genes and genomes (Tang et al., 2008). The study of orthologous and paralogous genes, contributes to the understanding of speciation processes. In many cases, candidate gene approaches may help elucidate the function of genes in related species. However, gene functions cannot always be extrapolated from structural orthologies (Irish and Litt, 2005).

Phenotypes, phenotypic plasticity and phenotypic spaces

Organisms are composed by their most obvious characters i.e. tomatoes and red skin of the fruit, the archetypes. These characters are the pillars that sustain the development of ideas around discrete elements (Figure 1). A step further into the description of nature lays on the secondary or minority components, those that contribute to the fine tuning of the system. Eventually, the aggregation of major and minor characters (phenotypes) leads to an accurate depiction of nature.

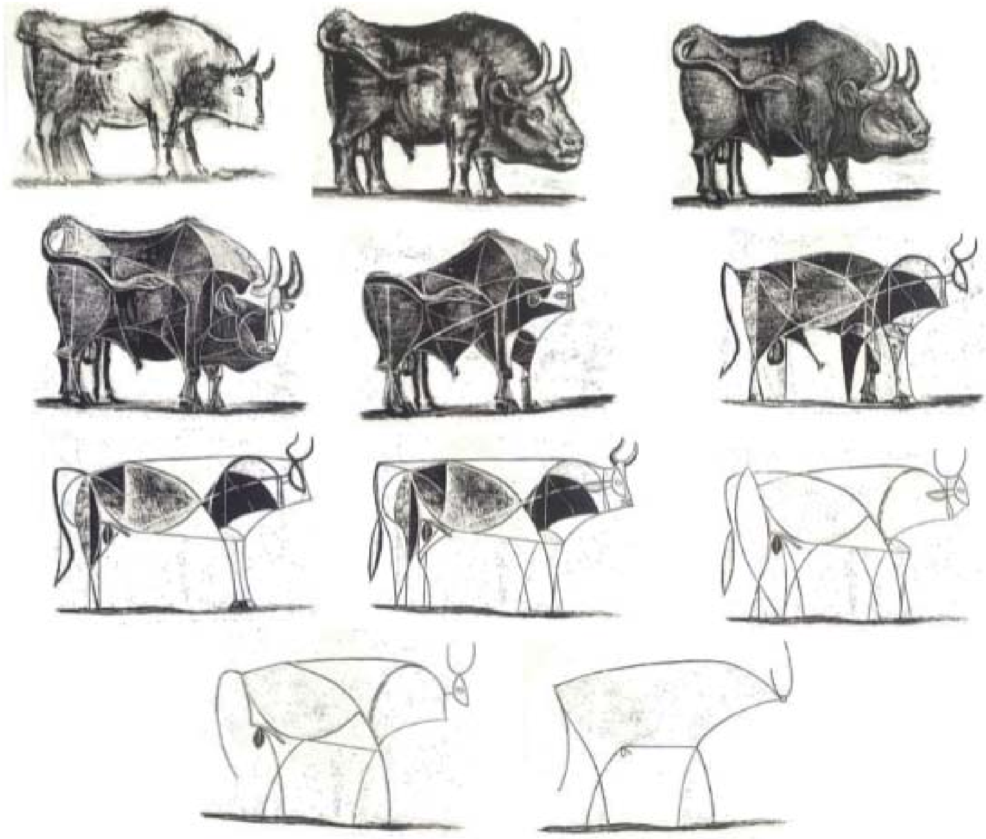

Figure 1. Picasso serial drafts to the ultimate representation of a bull by essential lines (https://www.curistoria.com/2016/04/la-destruccion-del-toro-por-picasso.html)

Phenotypes are the observable properties of organisms which include development, behaviour, appearance and composition. The expression of phenotypes depends on both, the coding potential of the organism and its interaction with the environment. A primary question in biology is what makes a species or a population different from each other. The ultimate goal in genetics studies is to associate phenotypes to the gene or genes underpinning its production. Although several causes might affect the genotype phenotype relationship apart from the genes/alleles related to the trait like the cellular environment, mechanical forces, symbionts, external molecules, temperature, epigenetic imprinting and individual background (Orgogozo et al., 2015).

Phenotypic variation is caused by the environmental (biotic and abiotic) factors that affect the expression of genes and allelic differences. This variation in the response to external factors leads to a phenotypic plasticity which can be studied from perspectives of developmental genetics and evolutionary biology (Sultan, 2005). The phenotypes that respond to environmental changes may have several layers of interaction including signal reception and transduction, transcriptional control and structural gene differences. Hence, phenotypic plasticity is the result of the activation or repression at the transcriptional level of pathways/genes involved in a particular trait (Sultan, 1995; Pigliucci, 1997; Price et al., 2003; de Kroon et al., 2005; Sultan, 2005).

At the species or community level, phenotypic plasticity may be the result of multiple alleles. It can be argued that the evolution of phenotypes is constrained by essentially two causes, the genetic coding potential and the natural selection (Pigliucci, 2007). Let along the physical laws which constrain the development of any organism (Shipley et al., 2006). The application of quantitative studies has led to the development of the concept of phenotypic spaces as a result of genetic and natural selection constrains (Pigliucci, 2007). An example of a quantitative phenotypic space in a species is the weight of dogs, from 1.5 kg of a Chihuahua to 80 kg of a Great Dane (Boyko et al., 2010).

Phenotypic traits can be considered as single elements such as metabolite content, or complex attributes such as colour or scent. Depending on the analytical techniques used, some phenotypic traits can broaden and a single phenotypic trait such as red colour can be represented numerically in several variables (L*, a*, b*, Cab* and hab) (Gupte, 2010). In the case of scent, a “fruity” scent can be identified with an array of different metabolites in distinct quantities (Lopez Pinar et al., 2017). For identifying and quantifying all metabolites included in such samples, omics approaches are required.

Volatile Organic Compounds (VOCs)

Volatile Organic Compounds (VOCs) are molecules with a low boiling point and high vapour pressure. These molecules are ubiquitous and they have been assigned multiple functions. VOCs are synthesized by bacteria, fungi, plants and animals (Arimura et al., 2001; Pichersky and Gershenzon, 2002; Schulz and Dickschat, 2007; Fontana et al., 2009; Cornu et al., 2011; Amo et al., 2013).

Volatiles are the basics of the usage of aromatic plants as flavourings and preservatives. Furthermore, VOCs present in essential oils have long been used as medical remedies for their spasmolytic, anti inflammatory, anticonvulsant, cholagogic, bronchodilatory, analgesic, expectorant and antimicrobial activities (Maffei et al., 2011). They are also known for their mosquitocidal and nematicidal effects (Momin and Nair, 2001).

The largest source of VOCs on earth are plants, with isoprene being by far the most abundant VOC (J. Nganga, 2001). A wide range of VOCs are synthesized and emitted to the troposphere. VOCs are classified as plant secondary metabolites. In general, they can be considered as infochemicals mediating interactions in ecosystems. They have numerous functions in plants like direct defence, indirect defence or pollinator attraction (Maffei et al., 2011). The complete set of VOCs emitted by organisms is known as its volatile metabolome, volatome or volatilome (Bicchi and Maffei, 2012).

Factors affecting scent emission and composition in flowers

Among specialized scientists, the high variability found in plant volatilomes is generally known. This variability affects both the composition and quantity of VOCs emitted. There are several factors that cause variability in measurements. For instance, VOCs are highly reactive in the troposphere and depending on their chemical nature, their life times range from minutes to months (Lerdau et al., 1997; J. Nganga, 2001). Additionally, allelic forms can affect the number and quantity of VOCs emitted. Furthermore, several physiological status can condition VOCs emission such as developmental stage of the tissue or organ analysed (Manchado Rojo et al., 2012; Ruíz Ramón et al., 2014). In addition, the time of the day of sampling or the pollination status also play a key role in the composition of scent (Rodriguez Saona et al., 2011).

Numerous studies have indicated changes in VOCs composition due to interactions of plants with insects, microorganisms and even with other plants (Schiestl, 2010; Bruce and Pickett, 2011; Galen et al., 2011; Caruso and Parachnowitsch, 2016; Groen et al., 2016; Martínez Medina et al., 2017). With regard to the importance of VOCs quantities, plants attacked by herbivores can emit 2.5 fold more quantity of VOCs than non attacked plants (Vuorinen et al., 2004). Even the egg deposition of insects on plants can increase the emission of certain VOCs (Beyaert et al., 2012) and attracting combinations of VOCs have been proved to remain efficient over a 1000 fold range (Riffell et al., 2009).

VOCs emission varies greatly during the day, with emitting peaks 10 fold higher than the average production (Cape, 2003; Maffei et al., 2011). In field sampling, ecological interactions in communities should also be taken into account (Farré et al., 2012), together with environmental conditions such as temperature, light or humidity (Sagae et al., 2008).

Finally, the degree of volatility of molecules depends on their chemical features i.e. saturation of carbon double bonds, oxygenation, hydrocarbon architecture, etc (Maffei et al., 2011). The chemical characteristics of VOCs and the analytical techniques used can affect the results obtained (Salem et al., 2016).

Scents as characteristic phenotypes for species clustering

Determining phylogenetic relationships relies on genomics, genetics, phenotypes and field data to reconstruct the evolution of species. During processes of speciation some hybridizations between "intermediate" individuals may happen and "final" phenotypes matching phylogenetical categories can be difficult to find in some cases (Vargas et al., 2009; Wilson and Hudson, 2011; Mann et al., 2017).

Constitutive scent profiles of species are the result of VOCs combinations mediating successfully in mutualist attraction and antagonist repellence. Scent profiles are blends characterizing species and some chemical ecological studies have used them for clustering different species according to their phylogenies (Hodgkison et al., 2013; Mann et al., 2017).

The development of tools which allow to easily characterize species and increase the number of samples analysed will allow the improvement of phylogenetical studies based on the study of phenotypic spaces and the determination of core volatile metabolomes.

Genetic structure of scents emission

The variety of VOCs found in flowers has been inventoried in more than 1700 (Knudsen et al., 2006). The analyses of scent profiles in rice, rose, petunia, tomato, peach or strawberry (Bradbury et al., 2005; Spiller et al., 2010; Klahre et al., 2011; Zorrilla

Fontanesi et al., 2012; Rambla et al., 2013; Sánchez et al., 2013) indicate remarkable efforts in delimiting the genetic structure of a complex trait. Despite these progresses, there is room for a lot of improvement regarding the identification of allelic differences and natural variation of genotypes related to volatilomes.

Main biochemical pathways conducting to the biosynthesis of plant VOCs are illustrated in Figure 3 (Maffei et al., 2011). The genetic structure of volatile scent emission comprises structural genes involved in the synthesis of intermediate metabolites and end products. But it also includes the transcription factors involved in activation or repression of scent synthesis and transporters involved in both intra cellular transport of metabolites and export of VOCs outside the cells (Adebesin et al., 2017). Thus, mutations or changes in genes codifying for the end product (VOC) will correspond to Mendelian segregations of simple hereditary characters, or epistatic segregations comprising a small number of genes. The effect of these isolated mutations can lead to changes in punctual VOCs emission (Dudareva et al., 2003; Spiller et al., 2010; Byers et al., 2014b).

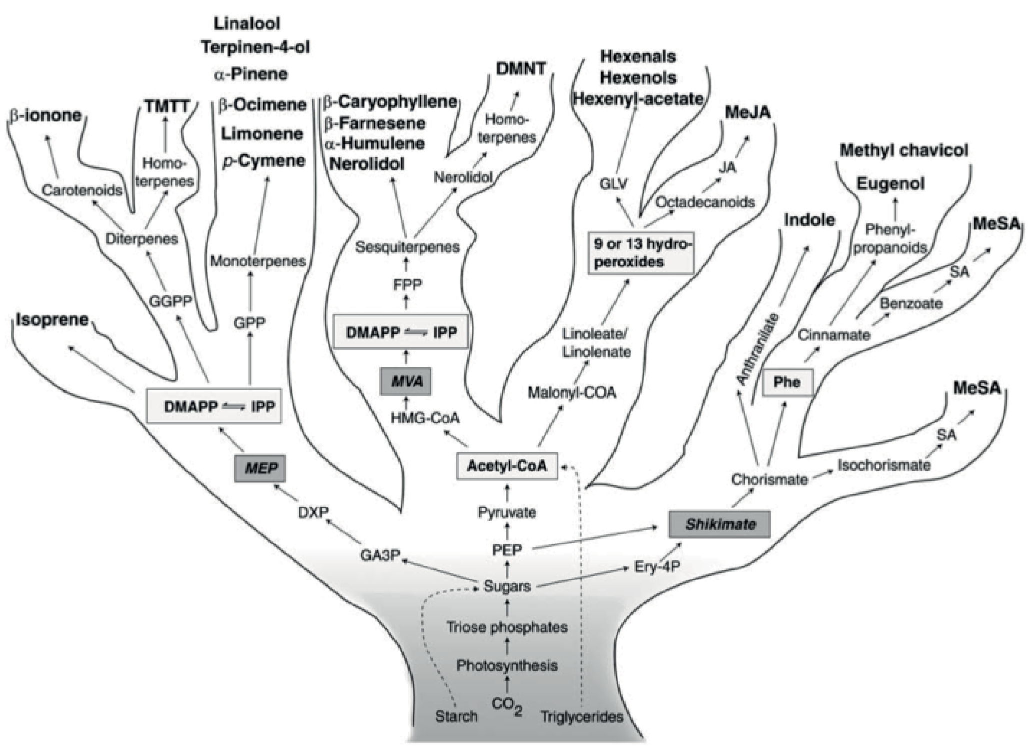

Figure 2. Plant volatiles biosynthetic tree (from: Maffei et al., 2011)

A different situation is found when mutations affect the expression of regulatory genes. The knockout of a transcription factor can lead to the loss or decrease of emission of several VOCs (Verdonk et al., 2005; Klahre et al., 2011). For instance, it has been described in *Petunia hybrida* that *EMISSION OF BENZENOIDS II* (*EOBII*) controls the transcription of both phenylpropanoid scent related structural genes and the shikimate pathway regulating *MYB* factor *ODORANT1* (*ODO1*) (Spitzer Rimon et al., 2010; Spitzer Rimon et al., 2012). However, the metabolic pathways leading to the production of such a diverse array of components and the genes associated to these routes are scarcely known (Muhlemann et al., 2014).

Furthermore, quantitative trait *loci* (QTLs) have been reported to contribute to the emission of scent in some cases. For instance, *ODO1* is one out of the two QTLs located in *Petunia* involved in the scent release of blends (Klahre et al., 2011). Additionally, two different QTLs are involved in the distinctive scent of Malaysian landraces of rice which has a remarkable economic importance (Golestan Hashemi et al., 2015). Moreover in ripe tomatoes, quantitative contributions of 21 QTLs are related to the emission of 29 VOCs (Wang et al., 2015). Contrastingly more complex structures have been revealed in cowpea, as a total amount of 63 QTLs have been identified as being involved in the emission of 22 VOCs (Andargie et al., 2014).

Scent release in flowers increases after anthesis. It has broadly been reported the interrelation of flower development and scent emission, with several traits such as pistil and stamen length and colour being co regulated (Colquhoun et al., 2011; Klahre et al., 2011; Van Moerkercke et al., 2011; Manchado Rojo et al., 2012; Hermann et al., 2013; Zu et al., 2016).

Another possibility affecting volatile metabolomes are mutations in genes codifying the machinery involved in VOCs release from cells, such as transporters or cuticle and cell wall lining (Widhalm et al., 2015; Raguso and Gottsberger, 2017; Tissier et al., 2017).

Indirectly affecting the selection of volatilomes is the effect of changes in the ultimate enzymes substrate. Some enzymes produce *in vitro* more than one metabolite, being able to react to different substrates *in vivo* (Effmert et al., 2005). This is known as enzyme promiscuity and leads to different reactions than these enzymes evolved for (Khersonsky et al., 2006).

Genetic models to study floral scent

Several model organisms are used for the study of both the genetic structure of floral scent production and interactions of plants with other organisms by means of VOCs release. Numerous works have been reported studying the emission of scent in *Petunia* spp. describing the transcriptional regulation of phenylpropanoids (Spitzer Rimon et al., 2010; Spitzer Rimon et al., 2012), the effect of temperature on the emission of VOCs (Sagae et al., 2008; Cna'ani et al., 2014), the circadian regulation of VOCs emission (Dudareva et al., 2000; Fenske et al., 2015; Fenske and Imaizumi, 2016), the relevance of structural genes on the emission of scent (Amrad et al., 2016) and the corregulation of pigmentation and scent emission (Cna'ani et al., 2015). Within the genus *Petunia* there are species which are preferentially pollinated by bee visitation (*P. integrifolia*) and by hawk moth visitation (*P. axillaris*) (Hoballah et al., 2005). Important efforts have been carried out in the analysis of scent emission related to pollinators behaviour (Hoballah et al., 2005; Klahre et al., 2011; Kessler et al., 2013). Belonging to the same family of tobacco, tomato, potato, aubergine and peppers, Petunia is taken as a relevant model organism.

Monkeyflowers belong to the genus *Mimulus* spp. which has served as a model in genetic studies on microgeographic adaptation (Hendrick et al., 2016), genome wide variation studies based on geographic cline analysis (Stankowski et al., 2017) and pollinator behaviour mediated by floral volatiles (Byers et al., 2014b; Byers et al., 2014a).

Another plant which has been broadly used in studies related to floral scent production is *Clarkia breweri.* It has a strong and sweet fragrance which consist of 8 to 12 VOCs (Wang et al., 1997). It has served as a model for understanding the developmental modulation of the emission of monoterpenes, specially linalool (Pichersky et al., 1994; Dudareva et al., 1996) and phenylpropanoids (Dudareva et al., 1998). Seminal works with *Clarkia breweri* isolated enzymes and genes responsible of the biosynthesis of common VOCs, such as linalool, methyl(iso)eugenol, benzylacetate, methyl salycilate and methyl benzoate (Dudareva et al., 2000).

The genus *Antirrhinum* has been used in studies of natural variation. *Antirrhineae* are distributed throughout Europe, the United States, and North Africa (Sutton, 1988). The species vary broadly in ecology and morphology, being adapted to rather different and extreme habitats and serve as a model for studies on development and natural variability. The popular ornamental snapdragon *Antirrhinum majus* has been used as a model plant in genetics since the early 20th century (Schwarz Sommer et al., 2003b). Its diploid inheritance, ease cultivation and remarkable variation in morphology and flower colour stimulated its use as a model organism.

The *Antirrhinum* species studied are able to form fertile hybrids between each other and with *A. majus*, thus allowing the identification of genes underlying their differences (Langlade et al., 2005). Laboratory lines of *A. majus* were produced from cultivars and an ample collection of mutants is available (Hudson et al., 2008). Traditionally, two independent inbred lines have been used for *Antirrhinum* research. The 165E line originated from the John Innes centre and the Sippe50 line from Germany. These lines have been used to create a map that allows the identification of mutations by crossing (Schwarz Sommer et al., 2003a). There are more than 25 wild species showing a remarkable degree of natural variation in traits such as floral size, colour, growth or trichomes. One of the species is *A. linkianum* (Boiss. & Reuter) which is an endemism distributed in north western areas of the Iberian Peninsula. In Spain is a threaten species, mainly for the loss of its habitats (Bañares et al., 2010). It grows in rocky and dune systems and its main pollinators are hymenopters and lepidodopterans (Comba et al., 1999).

Some studies have reported a regression to parental phenotypes in hybrids within the genus (Khimoun et al., 2011; Wilson and Hudson, 2011). Not many works have studied in detail the volatilome of *Antirrhinum* sp. (Suchet et al., 2010). Analysis of scent profiles in *Antirrhinum* species can enlighten the reasons underlying the parental phenotype regression under the shelter of the relationships between mutualists, antagonists and scents (Riffell et al., 2014; Junker and Parachnowitsch, 2015).

The effect of VOCs on insects

Insect choices for floral visits are modulated by several characters such as scent, colour, iridescence, morphology and rewards (nectar and pollen) (Glover and Martin, 1998;

Jones and Reithel, 2001; Moyroud et al., 2017; Bailes et al., 2018). The effect of individual floral traits on flower visitors depends on the context and they can interact additively or synergistically. For instance, the interaction between floral scents and colours, morphology and rewards has been illustrated. Hence multimodal approaches are required for studying the effect of scent profiles on insects preferences and establish possible mechanistics hypothesis of natural selection (Junker and Parachnowitsch, 2015).

The olfactory system of insects is designed in such a way that they can differentiate, from the vast background noise, the emission of one to several VOCs emitted from a source (Bruce and Pickett, 2011).

In the context of pollinator attraction by floral scents contrasting evidences have been found. On one hand, key VOCs have been shown as essential for triggering flower visits (Chen et al., 2009). On the other hand, complete scent profiles or subsets have been demonstrated as required for pollinator attraction (Riffell et al., 2009). In general, specialist plant pollinator relationships are often modulated by single key VOCs whereas in the case of more generalist pollinators, patterns in scent emission are more variable (Junker and Parachnowitsch, 2015).

Remarkable is the case of plant species pollinated by necrophoric insects like *Helicodiceros mucscivorous*, *Rafflesia* sp. or *Amorphophallus titanum* that emit rotten meat like scent to attract them. Combinations of sulphur containing VOCs seem to be essential in the process (Statheropoulos et al., 2007).

Antagonists play an important role in the natural selection of plants. Flowers are attacked by a number of herbivores and scents are part of the plant defence machinery. Moreover, plant scent profiles are used by herbivores for host identification. Although there have been found responses of pests to single VOCs, stronger behavioural responses were found with mixes of volatiles (3/10 VOCs) (Aharoni et al., 2005; Galen et al., 2011). Furthermore, relative proportions of these mixes have proved to be essential for insect response (Webster et al., 2010; Bruce and Pickett, 2011). Besides, there are also evidences of complex blends of VOCs acting as antimicrobials (Del Giudice et al., 2008).

Noteworthy is the case of natural enemies of plant antagonists attracted by VOCs emitted from plants. These VOCs can be used by natural enemies to locate their preys and composition of blends and quantities seem to be important in the process (Song and Ryu, 2013; Wason and Hunter, 2014).

Pests and pollinators: thrips and bumblebees

Thrips belong to the order *Thysanoptera* and are minute insects. The thrips species *Frankliniella occidentalis* is considered a world wide spread pest which affects more than 200 host plants (Campelo Rodríguez et al.). Despite acting as a pollinator for some wild species (Eliyahu et al., 2015), in the context of crops it is a major pest and its damages are produced by feeding, oviposition and transmission of diseases (Morse and Hoddle, 2006). Plant damages can be produced in both vegetative and reproductive organs. It affects a broad range of crops, from vegetables to fruits and it is the main vector of the tomato spotted wilt virus (TSWV). With respect to flowers, pollen feeding thrips, such as *Frankliniella occidentalis,* have been reported as having negative effects on pollination, fruit quality, crop yield and plant fitness (Kirk, 1987; Rosenheim et al., 1990; Coll et al., 2007; Kirk, 2009). Important efforts are made in the control of this pest, including the use of pesticides (Puinean et al., 2013) and natural enemies (Rocha et al., 2015).

Among the order *Hymenoptera* there are important families of insects including bees, ants, wasps and sawflies. Within the order, there are both solitary and social species. Bumblebees (*Bombus* spp.) are important pollinator species for both wild plants and crops. Bumblebees feed from nectar and pollen and are broadly used for the pollination of agricultural productions such as tomato, *Cucurbita* spp., cocoa or vanilla (Garibaldi et al., 2011; Morse et al., 2012). Among bumblebees, buff tailed bumblebee (*Bombus terrestris*) is the main species of eusocial bumblebees distributed in Europe, although in southern areas is not naturally found in the wild due to issues on temperature management (Goulson, 2014). It is a well studied species, because of its economical relevance.

Methodologies of detection and quantification of VOCs

The analytical advances have allowed the improvement of the detection and analysis of these metabolites. Headspace gas chromatography coupled to mass spectrometry (GC MS) allows efficient qualitative and quantitative analysis of VOCs, although other techniques such as electronic noses are also available for VOCs analysis (Cui et al., 2018a).

VOCs analysis has reached in some cases the biological sensitivity threshold (Splivallo et al., 2007). Appropriate quantification of metabolites content requires the usage of commercial standards of each VOC analysed (Callejón et al., 2008). This premise entails several drawbacks like economical costs when analyses are based on non targeted metabolomics (D'Agostino et al., 2014), the lack of commercial standards (Roessner et al., 2000; Raffo et al., 2018) and the coexistence of isomers eluting at equal retention times.

Due to the discussed variability in VOC qualitative and quantitative emission, volatilome studies are complex. Therefore, most studies of VOCs have focused their attention on one to few different metabolites, and there are fewer studies focusing on entire scent profiles or volatilomes. Given the known importance of VOC proportions in scent blends (Bruce and Pickett, 2011) and the relative unimportance of quantities regarding VOCs bioactivity (Riffell et al., 2009), additional methodologies for volatiles quantification can be used. Despite the derived technical inaccuracies that may be committed by quantifications not using chemical standards, reflecting the relative proportion of VOCs when screening volatile metabolomes can be the most important objective in some studies.

Phenotyping core and inducible metabolism

Although families of metabolites can be very varied, only small fractions of them can be synthesized by a given genotype. This fact is known as specialized metabolism and is, by definition, the most diverse metabolism (Chen et al., 2011). Specialized metabolism is cause and effect of natural selection. In this context, constitutive and non constitutive phenotypes/metabolism have a direct implication in species survival.

While core or constitutive elements are those that are evenly present among different entities under the same category, non constitutive elements are induced by environmental inputs and/or physiological stages. These inputs can be caused by a number of stresses such as salinity, light, temperature (Sagae et al., 2008), herbivore feeding (Amo et al., 2013) or systemic acquired resistance (Liu et al., 2010). Their functions can be essential for the survival of organisms, acting directly as defence compounds or as signals activating metabolic routes (Reichling, 2018), on the other hand, their constitutive expression can be prejudicial for plants in the absence of elicitors (Strauss et al., 2002).

The development of mass spectra libraries and related programs has eased the automatic GC MS data annotation (Chaparro Torres et al., 2016). Recent publications have progressed the automatic establishment of core volatile metabolomes (Domingo Almenara, 2016). Despite these progresses, the statement of parameters determining what is the core volatilome and which are non constitutive metabolites is mostly done manually.

In the study of scent phenotypic spaces, both core metabolomes and non constitutive volatiles reflect the plasticity of the trait. The development of tools which allow increasing the number of samples and stablishing the thresholds which delimit what are considered inducible and constitutive volatilomes will ease the analysis and reliability of studies of such a complex trait. A general agreement on the treatment of GC MS results would help in the comparison of inter studies outcomes. Detailed scent phenotyping will help studies aiming to analyse the genetic structure of scent in plants.

Ideally, for establishing the core metabolome of a set of samples they should be taken under the same developmental stages. Moreover, circadian variations of metabolites synthesis should be taken into account when sampling and obviously, the number of samples analysed plays an important role in the conclusions obtained. Growing conditions affect the core and inducible metabolism. Thus, depending on how, when and where plants are grown results can vary. It is known that under field conditions where plants are exposed to more environmental inputs than in vitro cultures, metabolomes are more varied.

Constitutive scent blends are the result of selection pressures exerted by mutualists, such as pollinators, and antagonists, such as pests. Studies on VOCs mediating interactions of plants with pests a pollinators bring to light the characters under pressure for selection. These selection pressures constrain scent profiles and together with genomes coding potential, determine scent phenotypic spaces.

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Objectives of the Thesis

The general aim of this PhD dissertation was to uncover the genetic and evolutionary basis of volatile emission in *Antirrhinum* and to obtain a better understanding of plant pollinator/pest interactions mediated by volatiles. This general objective confronted us with some methodological and technical questions to be tackled. Each chapter of this PhD dissertation responds to different objectives which together, assess the main objective of the thesis.

Chapter I

∴ Characterize floral scent emission of *A. linkianum, A. tortuosum, A. cirrigherum, A. latifolium, A. meonanthum, A. braun blanquetii, A. barrelieri, A. graniticum* and *A. majus* (165E and Sippe50).

∴ Analyse the effect of the developmental stage of flowers on scent emission (quantities and composition).

Chapter II

∴ Analyse the genetic structure of the emission of methyl benzoate, methyl cinnamate, acetophenone and ocimene in *A. majus* and *A. linkianum*.

∴ To study the molecular differences leading to the lack of emission of methyl benzoate in *A. linkianum* following a candidate gene approach (*A. majus BAMT*).

Chapter III

∴ To compare the effect of several semi quantitative methods on the proportions of VOCs within scent profiles and between samples.

∴ Explore the usage of nearest components and single calibrators for semi quantifications.

Chapter IV

∴ To develop an R package that automatically analyses GC MS output files by using either Agilent Technologies standard output or CSV files.

∴ To develop a tool that determines what is considered the core metabolome and non constitutive volatile metabolome.

∴ To develop a tool that uses CAS numbers instead of names of compounds and facilitates the analysis and comprehension of data.

Chapter V

∴ To analyse the preferences of bumblebees and thrips for different scent profiles.

∴ To obtain key volatiles mediating plant insect interactions.

∴ To indicate volatiles that may be relevant within blends in the interaction between plants and insects.

Chapter I

Phenotypic Space and Variation of Floral Scent Profiles during Late Flower Development in Antirrhinum

frontiers in Plant Science

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Phenotypic Space and Variation of Floral Scent Profiles during Late Flower Development in *Antirrhinum*

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The genus *Antirrhinum* comprises about 28 species with a center of origin in the Iberian Peninsula. They show an important diversity of growing niches. We have performed a comprehensive analysis of scent profiles in eight wild species, *Antirrhinum linkianum*, *A. tortuosum*, *A. cirrigherum*, *A. latifolium*, *A. meonanthum*, *A. braun-blanquetii*, *A. barrelieri*, and *A. graniticum*. We used also two laboratory inbred lines *A. majus*, 165E and Sippe50. We identified 63 volatile organic compounds (VOCs) belonging to phenylpropanoids, benzenoids, mono- and sesquiterpenes, nitrogen-containing compounds, and aliphatic alcohols previously described in plants. Twenty-four VOCs were produced at levels higher than 2% of total VOC emission, while other VOCs were emitted in trace amounts. The absolute scent emission varied during flower maturation and species. The lowest emitting was *A. meonanthum* while *A. tortuosum* had the largest emissions. Species were clustered according to their scent profiles and the resulting dendrogram matched the current species phylogeny. However, two accessions, *A. majus* Sippe 50 and *A. braun-blanquetii,* showed development-specific changes in their VOC composition, suggesting a precise control and fine tuning of scent profiles. Cluster analysis of the different scent components failed to identify a specific synthesis pathway, indicating a key role of scent profiles as blends. There is considerable degree of chemodiversity in scent profiles in *Antirrhinum*. The specific developmental stage plays an important role in scent quantitative emissions. The relative robustness of the bouquets could be an adaptation to local pollinators.

Keywords: floral scent, flower development, anthesis, phylogeny, biodiversity, chemodiversity, *Antirrhinum*

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INTRODUCTION

The interaction between plants and other organisms is thought to be mediated by a complex set of traits among which the emission of chemical compounds plays a key role. The so-called plant volatiles are one of the most diverse set of molecules. Plant volatile emission can be classified according to the source of emission, i.e., leaves, flowers, and roots. And it can also be the result of certain reactions such as defense against herbivores or parasites. The emission of scent by flowers is a cue that helps to make floral sexual organs attractive to potential pollinators, but also works in parasite deterrence (Schiestl, 2010). In most flowers, floral scent is emitted by petals and stamens (Dudareva et al., 1996; Verdonk et al., 2003; Scalliet et al., 2006). Although over 1700 volatile organic compounds (VOCs) are described in plants, the actual composition of floral scent is not fully explored in most plant species (Knudsen et al., 2006).

Petal and stamen development in *Antirrhinum* and many other species is directly controlled by B function organ-identity genes (Egea Gutierrez-Cortines and Davies, 2000; Causier et al., 2010). The B function genes in *Antirrhinum* are the MADS-Box genes *DEFICIENS* and *GLOBOSA*. Their expression is required in a quantitative manner to attain fully developed petals and stamens (Bey et al., 2004; Manchado-Rojo et al., 2012). Floral scent emission is a late process starting shortly before anthesis in a variety of species (Knudsen et al., 2006), but its quantitative levels are regulated upstream by the B-function genes (Manchado-Rojo et al., 2012). Scent production varies after anthesis showing an increase in production till a point when sharp decreases are caused by flower aging and/or pollination (Pichersky et al., 1994; Ruiz-Ramon et al., 2014).

Antirrhinum, a genus native to the western Mediterranean region, comprises a monophyletic group with approx. 28 species (Liberal et al., 2014), traditionally assigned to the three morphological subsections or clades: *Kicksiella*, *Antirrhinum*, and *Streptosepalum* (Rothmaler, 1956; Webb, 1971; Sutton, 1988). The *Antirrhinum* flower has an occluded corolla (Vargas et al., 2010; Guzmán et al., 2015). It is apparently specialized in bee pollination as bees such as *Rhodanthidium sticticum* is the main pollinator of *A. microphyllum*, (Torres et al., 2003), and seven types of bees account for over 90% of the pollination visits in *Antirrhinum charidemi*, *Antirrhinum graniticum*, and *Antirrhinum braun-blanquetii* (Vargas et al., 2010). Despite the diversity the composition of the *Antirrhinum* genus floral scent, like that of many other plants, is basically unexplored and only *A. majus sp pseudomajus* and *A. striatum* have been analyzed with detail (Suchet et al., 2010).

In this work, we present a comprehensive analysis of floral VOCs in eight wild *Antirrhinum* species: *Antirrhinum linkianum*, *A. tortuosum*, *A. cirrigherum*, *A. latifolium*, *A. meonanthum*, *A. braun-blanquetii*, *A. barrelieri*, and *A. graniticum*. We have also used two laboratory inbred lines, *A. majus* 165E and Sippe50. These lines have been used for genetic studies, development of an *Antirrhinum majus* genetic map and for genetic transformation (Schwarz-Sommer et al., 2003, 2010; Manchado-Rojo et al., 2012, 2014). We identified at least 63 VOCs produced at one stage after anthesis and before petal senescence. Each species had a unique blend of VOCs, and tended to show a robust profile except for two species. The scent profiles allowed a cluster reconstruction that matched published phylogenies based on molecular markers indicating a uniqueness of scent signature for each species that may have implications for local adaptation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

We obtained eight wild species of *Antirrhinum* and two laboratory inbred lines (**Table 1**). The wild species include species of subsection *Antirrhinum*, series *Majora: A. barrelieri, A. cirrhigerum, A. graniticum, A. latifolium,* and *A. tortuosum* (Mateu-Andres and De Paco, 2005) as well as the two only members of subsection *Streptosepalum, A. braun-blanquetii* and *A. meonanthum* (Feng et al., 2009) (**Figure 1**). We also used two laboratory inbred lines, *A. majus* Sippe50 isolated at the beginning of the 20th century in Germany (Stubbe, 1966) and *A. majus* 165E developed at the John Innes Centre (Harrison and Carpenter, 1979; Sommer and Saedler, 1986). The geographical distribution of the species surveyed includes the Pyrenees, northern Spanish coast, Portugal, southern Spanish coast, and northern Africa (**Figure 1**). Plants were grown under standard greenhouse conditions using large pots of 3–5 l to increase the number of flowers obtained (Weiss et al., 2016). Four to five plants for each species and line were propagated and flowers were sampled randomly from these plants for further analysis.

TABLE 1 | Name and origin/supply of *Antirrhinum* species.

Species name	Origin
Antirrhinum barrelieri Boreau	Vendrell, Tarragona Province, and Spain
Antirrhinum braun-blanquetii Rothm.	Province of Oviedo, Picos de Europa, and Spain
Antirrhinum meonanthum Hoffmanns and Link	Penacova and Portugal
Antirrhinum latifolium Mill.	Ville Franche, Pyrenees, and France
Antirrhinum graniticum Rothm.	Unknown
Antirrhinum. linkianum	Supplied by Bot. Garden, University of Coimbra, Portugal
Antirrhinum cirrhigerum	Unknown, Spain
Antirrhinum tortuosum	Unknown, Spain
Laboratory lines	
Antirrhinum majus L. line 165E	Our stocks
A. majus L. line Sippe 50	Supplied by IPK Gatersleben

VOC Collection

Flower samples were taken daily during six days after flower opening and emitted volatiles were analyzed by dynamic headspace analysis (Raguso and Pellmyr, 1998). For each flower developmental stage, three randomly chosen, detached flowers were placed in 5% sucrose solution in transparent glass containers. Volatile sampling was performed over a 24-h period in a growth chamber (model E8; Conviron, Asheville, NC, USA) with a photoperiod of 12:12 light: dark conditions. Scent components were trapped with Porapak Q-filled glass syringes in a closed-loop scent collection system. Trapped volatiles were eluted from the adsorbent with dichloromethane.

Gas-Chromatography Mass Spectrometry

Trapped floral volatiles were analyzed by gas chromatography–mass spectrometry (GC-MS) as described (Dudareva et al., 2003). Data analysis and volatile identification was performed with the MSD ChemStation (Agilent Technologies) software. The compounds were identified by comparing mass spectra and retention time (RT) data with those of authentic standards for benzaldehyde, β-myrcene, 2-ethyl-1 hexanol, β-ocimene,

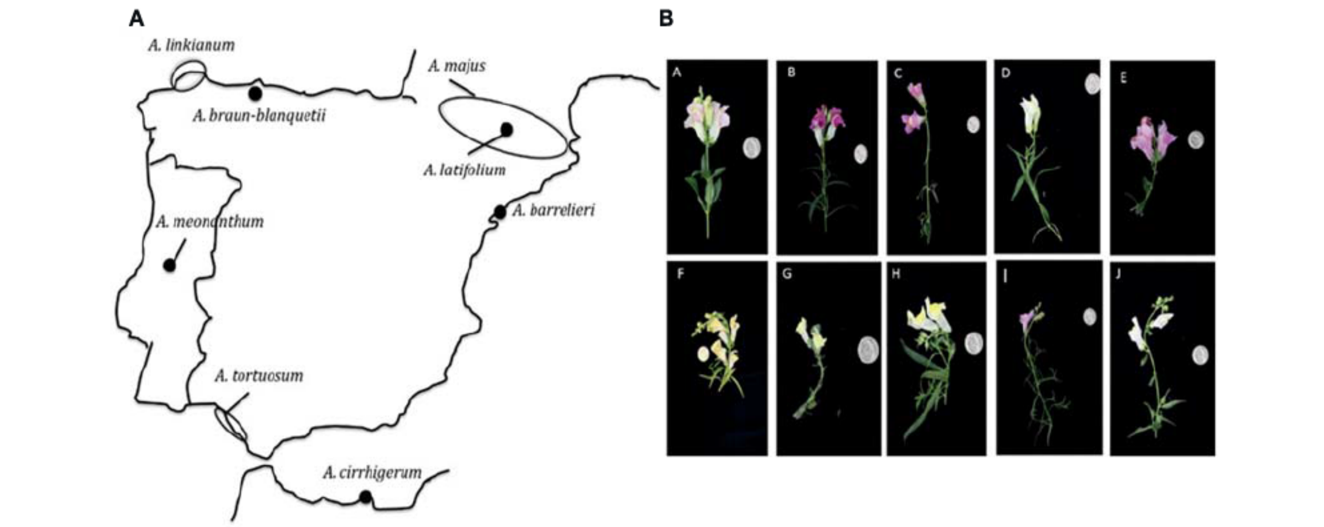

FIGURE 1 | Origin of *Antirrhinum* species used in the study. (A) Origin of seeds (filled circles) used in this study. For seeds of unknown origin, the area of distribution according to Vargas et al. (2009) is indicated (empty circle). *A. graniticum* is widely spread over central Iberian Penisula and the Western coast area, but absent in the North and East of the Iberian Peninsula (Vargas et al., 2010). **(B)** Flowers of the studied *Antirrhinum* species. A 25 cent USA-dollar is photographed for scale: (A) *A. majus* line 165E, (B) *A. majus* line Sippe 50, (C) *A. linkianum*, (D) *A. tortuosum*, (E) *A. cirrigherum*, (F) *A. latifolium*, (G) *A. meonanthum*, (H) *A. braun-blanquetii*, (I) *A. barrelieri*, and (J) *A. graniticum*.

acetophenone, methyl benzoate, linalool, and methyl cinnamate, supplemented with information from the NIST11 spectral library. The relative contribution of volatile compounds was calculated based on the integrated area of particular peaks relative to the total integrated peak area for the flower opening stages I = day 1, II = day 3, and III = day 5. Total volatile amount was calculated based on integrated peak area of a defined amount of the internal standard naphthalene. Total amounts are given as integrated area of peaks normalized to naphthalene/g fresh weight $(FW)^{-1}$ 24 h^{-1}. Supplementary Figure S1 shows one chromatogram of each species at stage III. The different volatiles in percentages can be found Supplementary Table S1.

Cluster Analysis and Principle Component Analysis of Volatiles

For hierarchical cluster analysis, the relative amounts of the 24 most abundant, major volatile compounds were used. We considered as major compounds those that accounted for equal or more than 2% of total amounts in the different flowering stages of the species or subspecies analyzed. The cluster analysis included the volatile profiles of flower opening stages I, II, and III as mentioned above. Clustering of species and developmental stages was achieved using R version 2.13.1, with Pearson correlation and average linkage serving as correlation and agglomeration methods.

Principal component analysis (PCA) was performed with absolute amounts of all VOCs (**Table 2**). Each sample collected was included in this analysis. To satisfy the assumption of linearity, absolute amounts were $\log 10(n+1)$-transformed prior to PCA. PCA with varimax rotation was performed with the prcomp command in the stats package in R version 2.13.1.

RESULTS

Phenotypic Space of Scent Emission

The emission of floral volatiles starts at late stages of petal morphogenesis requiring fully developed petals and anthesis (Manchado-Rojo et al., 2012; Muhlemann et al., 2012). We investigated the production of floral scent over a time span of six days after flower opening and identified a total of 63 based on NIST 11. There were 63 that matched VOCs previously identified in plants (**Table 2**). They belonged to the following chemical categories: phenylpropanoids, benzenoids, mono- and sesquiterpenes, nitrogen-containing compounds, and aliphatic alcohols (Knudsen et al., 1993). Amongst the compounds identified and found in a variety of plants and in *Antirrhinum* were benzenoids such as vanillin, o-acetanisole, methyl salicylate, anisole or cuminyl alcohol; isoprenes such as alpha pinene or terpineol. Phenylpropanoids included cinnamyl formate; fatty acid derivatives as aldehydes including octanal, decanal, nonanal or alcohols such as octanol or as acids. Flowers also emitted amines or nitrogen containing compounds such as methyl nicotinate, indole or indolicine 1,3,5,7-cyclooctatetraene, a non-classified compound.

We additionally found nine VOCs that had not been described previously as emitted by plant tissues (**Table 3**). They could be grouped into the classic set of benzenoids, phenylpropanoids, and fatty acid derivatives VOCs.

From the large dataset presented, there were 24 major compounds comprising more than 2% of the scent emission in the different species (**Figure 2** and Supplementary Figure S1). Among these we found benzaldehyde, acetophenone, ocimene, and 2-ethyl 1-hexanol in all the species analyzed, comprising very different percentages of the scent profile. At the other side of the spectrum, 1,4-dimethoxybenzene was present only in

TABLE 2 | List of volatile organic compounds (VOCs) identified in *Antirrhinum* and known to be biosynthesised by plants.

Plant emitted volatiles	CAS number	Retention time (RT)	% Probability
Benzenoid – Aldehydes			
Benzeneacetaldehyde	122-78-1	10.925	90
Benzaldehyde	100-52-7	9.9076	94
Benzaldehyde, 3-ethyl-	34246-54-3	13.316	95
Vanillin	121-33-5	17.488	90
Benzaldehyde, 4-ethyl-	4748-78-1	13.311	90
3,5-Dimethoxybenzaldehyde	7311-34-4	18.077	98
Benzaldehyde, 4-methoxy-	123-11-5	15.016	94
Benzenoid – Ketones			
Acetophenone	98-86-2	11.491	97
4-Acetylanisole	100-06-1	16.721	94
Ethanone, 1-(4-ethylphenyl)-	937-30-4	15.502	97
Benzenoid – Esters			
Benzyl Benzoate	120-51-4	22.924	98
Methyl benzoate	93-58-3	11.995	94
Benzoic acid, 3,5-dimethoxy-, methyl ester	2150-37-0	20.246	98
Methyl salicylate	119-36-8	13.934	97
Benzoic acid, 4-methoxy-, methyl ester	121-98-2	17.081	81
Benzoic acid, 2-butoxy-, methyl ester	606-45-1	13.934	97
Benzenoid – Ethers			
3,5-Dimethoxytoluene	4179-19-5	15.222	98
1,2,4-Trimethoxybenzene	135-77-3	16.973	94
Anisol	100-66-3	8.086	91
Benzene, 1,3,5-trimethoxy-	621-23-8	17.625	96
Benzenoids – Benzenes			
Benzene, 1,3-diethyl-	141-93-5	1.033	97
Benzene, 1,4-diethyl-	105-05-5	11.176	97
Benzene, 1,2-diethyl-	135-01-3	11.291	96
p-Xylene	106-42-3	6.959	95
Ethylbenzene	100-41-4	6.776	94
Benzene, 1,2,3-trimethyl-	526-73-8	9.826	92
Benzene, 1,4-dimethoxy-	150-78-7	13.294	96
Benzene, 1,2-dimethoxy-4-(2-propenyl)-	93-15-2	17.505	98
Benzenoids – Alcohols			
Benzyl Alcohol	100-51-6	10.679	95
3-Methoxy-5-methylphenol	3209-13-0	16.120	94
Cinnamyl alcohol	104-54-1	15.891	98
Benzenepropanol	122-97-4	14.547	98
Benzenemethanol, 4-methoxy-	105-13-5	15.502	95
Phenol, 4-(1,1-dimethylethyl)-2-methyl-	98-27-1	14.775	93
Phenol	108-95-2	9.540	74
Isoprenoids–Monoterpenes			
Myrcene	123-35-3	9.786	96
Ocimene	3779-61-1	11.002	98
Neo-allo-ocimene	7216-56-0	12.618	96
Linalool	78-70-6	12.058	97
Limonene	138-86-3	10.581	99
α-Pinene	80-56-8	8.476	95
Terpineol	98-55-5	13.849	90
Isoprenoids–Sesquiterpenes			
α-Farnesene	502-61-4	19.164	97
Nerolidol	7212-44-4	20.023	95

(Continued)

TABLE 2 | Continued

Plant emitted volatiles	CAS number	Retention time (RT)	% Probability
Phenylpropanoids – Alcohols			
Eugenol	97-53-0	16.784	98
Phenylpropanoids – Esters			
Methyl cinnamate	103-26-4	17.219	97
Cinnamyl formate	21040-45-9	16.692	98
Phenylpropanoids –Aldehydes			
Cinnamaldehyde	104-55-2	15.308	98
Fatty acid derivatives – Aldehydes			
Decanal	112-31-2	14.066	91
Nonanal	124-19-6	12.126	87
Hexanal, 2-ethyl-	123-05-7	8.951	81
Fatty acid derivatives – Ketones			
2-Pentadecanone, 6,10,14-trimethyl-	502-69-2	23.833	99
5-Hepten-2-one, 6-methyl- Methylheptenone	110-93-0	9.677	94
γ-Hexenol	928-96-1	6.656	91
Fatty acid derivatives – Alcohols			
1-Hexanol, 2-Ethyl,	104-76-7	10.576	90
Phenoxyethanol	122-99-6	14.346	95
Fatty acid derivatives – Acids			
Dodecanoic acid	143-07-7	19.885	91
Amines and other nitrogen containing compounds			
Indolizine	274-40-8	15.714	86
Indole	120-72-9	15.708	95
Methyl nicotinate	93-60-7	12.807	95
Benzyl nitrile	140-29-4	12.836	96
Diphenylamine	122-39-4	20.915	90
Non-classified			
1,3,5,7-Cyclooctatetraene	629-20-9	7.474	70

Retention times are approximated and consistent between all the chromatograms analyzed. % Probability column indicates the existing chances of success in identifying compounds for a given RT, according to the NIST11 database. Internal standard (Naphthalene, CAS no. 91-20-3) probability is over 95%.

A. braun-blanquetii, nerolidol was found in *A. braun-blanquetii* and *A. latifolium* and 5,9-dodecadien-2-one, 6,10-dimethyl was found in *A. meonanthum* and *A. graniticum*.

The complexity of the different suggested scent profiles in terms of number of VOCs emitted varied greatly. The most complex profile was exhibited by *A. braun-blanquetii* comprising 21 VOC compounds above 2% over at least one of the three developmental stages analyzed (**Figure 2** and Supplementary Figure S1). In contrast there were five accessions with a much simpler scent profile such as *A. linkianum* with 10 major VOCs followed by *A. cirrhigerum*, *A. majus* Sippe50 and *A. tortuosum* with 11 VOCs and *A. majus* 165E with 12.

In summary, the species analyzed appeared to show two distinct suggested profiles as those with relative low scent complexity lack irregular terpenes, fatty acid aldehydes and ketones, and nitrogen containing compounds.

Changes in Total and Relative Emission of VOCs during Flower Development

We analyzed the scent emission during a period of seven days. Flowers of all species produced scent during the entire sampling period. The average emission of scent during the period varied between species (**Figure 3**).The lowest emitting species was *A. meonanthum* while the species with larger levels of production corresponded to *A. latifolium* followed by the two *A. majus* 165E and Sippe50 inbred lines.

We analyzed the quantitative changes in emission of the different compounds throughout development (**Figure 4**). The two *A. majus* inbred lines used for many experiments in plant development and the wild species *A. latifolium* and *A. barrelieri* produced acetophenone as major volatile. They also showed comparable levels of methyl benzoate and ocimene emission. However, they differed in the emission of myrcene *by A. majus 165E* and methyl cinnamate by *A. majus S.50*. The profile of *A. latifolium* included ocimene, 3,5-dimethoxytoluene, benzeneacetaldehyde, and myrcene, while *A. barrelieri* emitted ocimene, cinnamyl alcohol, and myrcene.

There were three species, *A. linkianum*, *A. tortuosum*, *and A. braun-blanquetii* that emitted ocimene as major volatile. However, the rest of the volatiles were not in common as *A. linkianum* produced methyl cinnamate, myrcene, and 2-ethyl 1-hexanol. The scent profile of *A. tortuosum* included myrcene, acetophenone, and linalool while *A. braun-blanquetii* showed high levels of cinnamyl alcohol, 3,5-dimethoxytoluene, and methyl benzoate.

TABLE 3 | List of new VOCs identified in *Antirrhinum* and previously unidentified in plants.

New volátiles	CAS number	RT	Quality
Benzenoid – Ketones			
Acetophenone, 2′-hydroxy-	118-93-4	13.311	97
Benzenoid – Esters			
Benzenepropanoic acid, methyl ester	103-25-3	15.731	92
Benzenoids – Benzenes			
Benzene, 1-(1,1-dimethylethyl)-4-methoxy-	5396-38-3	14.775	94
Benzene, 1-ethenyl-3-ethyl-	7525-62-4	11.766	96
Benzenoids – Alcohols			
Phenol, p-tert-butyl-	98-54-4	15.645	97
Phenol, 2,6-dimethoxy-4-(2-propenyl)-	6627-88-9	20.606	97
Benzenethanol	60-12-8	12.338	93
Phenylpropanoids – Esters			
Cinnamyl acetate	103-54-8	18.180	97
Fatty acid derivatives – Alkanes			
Adamantane, 1,3-dimethyl-	702-79-4	12.378	90

Retention times are approximated and consistent between all the chromatograms analyzed. % Probability column indicates the existing chances of success in identifying compounds for a given RT, according to the NIST11 database. Internal standard (Naphthalene, CAS no. 91-20-3) probability is over 95%.

Finally, three species showed a different VOC as major compound. The major VOC in *A. cirrhigerum* was methyl benzoate, and emitted acetophenone, 3,5-dimethoxytoluene and ocimene. The scent profile of *A. meonanthum* was complex as its debut was dominated by 2-ethyl-hexanol but was taken over by benzene acetaldehyde. It also emitted acetophenone, cinnamyl alcohol, benzaldehyde, nonanal, and the nitrogen containing indole. The main component emitted by *A. graniticum* was cinnamyl alcohol, methyl benzoate, benzyl benzoate, benzaldehyde, and benzeneacetaldehyde.

Concerning the quantitative changes in emission during flower maturation, the quantities varied and the variance was high. This is probably due to temperature changes during flower maturation. Thus a general pattern of emission cannot be found for all the species.

Scent-Based Clustering of *Antirrhinum* Species and Robustness of Scent Profiles

To determine whether differences in scent emission between species are greater than emission differences between developmental stages within species, we collected volatile samples for the developmental stages I–III. Suggested volatile profiles for most of the species presented here, except *A. majus* line Sippe 50 and *A. braun-blanquetii*, clustered together for all flower developmental stages (**Figure 5**), demonstrating that the profile of the 24 major volatiles changed less between developmental stages than between the species. In case of *A. majus* Sippe 50 and A. *braun-blanquetii*, suggested scent profiles of different flower

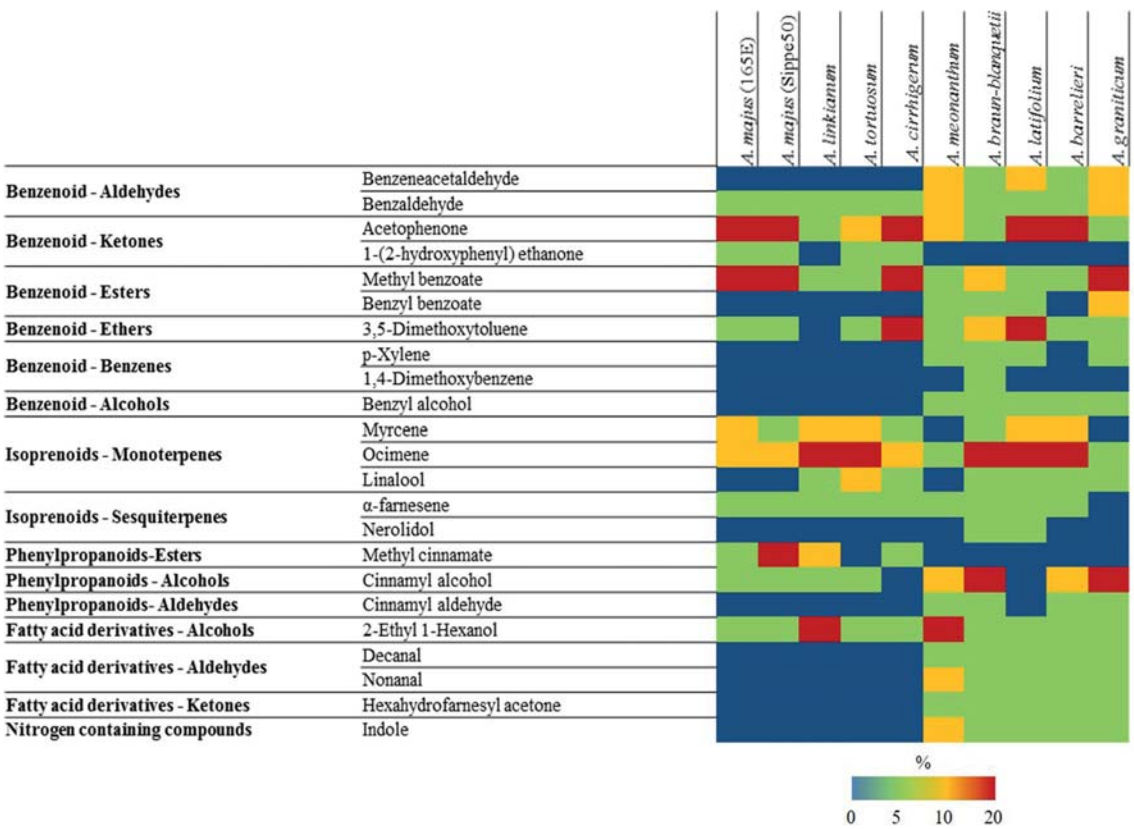

FIGURE 2 | Heat map with major VOCs (above 2% of total volatiles present in chromatograms) emitted by different species of *Antirrhinum*. Colors reflect the maximum level of emission (%) in stages I–III for each species. Key color emission: blue (0%), green (<5%), yellow (<20%), and red (>20%).

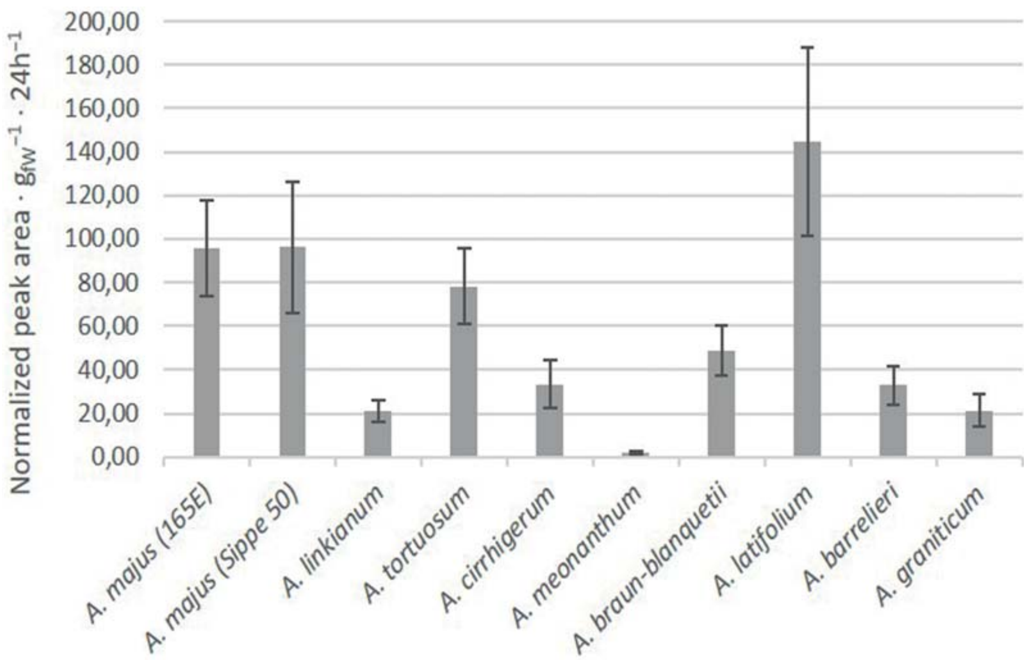

FIGURE 3 | Average emission of VOCs in the different accessions. Quantities are total integrated area normalized to naphthalene/g 24 h^{-1}. Error bars correspond to standard error.

ages clustered in different branches, indicating variations in the composition of fragrances during development. This highlights that sampling several developmental stages is a critical factor if the volatile profile is to be used for taxonomic interpretation.

The suggested volatile profiles of *A. meonanthum and A. braun-blanquetii*, both belonging to subsection *Streptosepalum*, build separate clusters from members of subsection *Antirrhinum*, with the exception of *A. graniticum*, which clustered with *A. braun-blanquetii*, separate from all other members of subsection *Antirrhinum*.

Within subsection *Antirrhinum*, except for *A. graniticum*, species branched into two main clusters. One of these two branches contained *A. linkianum* and *tortuosum*. Within the second major branch, *A. majus* and *cirrigherum* on one side and *A. barrilieri* and *latifolium* on the other side showed a closer relatedness.

Identification of Associated Odor Descriptors by PCA

To identify scent compounds that contribute to the variation in VOC profiles between species, we performed a PCA. We extracted four components that account for 82% of the variance in the data (**Table 4**). The first principal component, which explains 58% of the variance observed in scent emission between species, displays negative loadings for acetophenone and ocimene. The two compounds with the highest correlation to the second principal component were cinnamyl alcohol and 2-ethyl-1-hexanol. The third principal component contrasted the presence of cinnamyl alcohol with that of acetophenone, with a positive loading for cinnamyl alcohol and a negative loading for acetophenone. Lastly, the fourth principal component was highly correlated to methyl benzoate and ocimene. These data reveal that variance in volatile profiles between *Antirrhinum* species is caused by differences in emission levels of VOCs originating from different biosynthetic pathways, rather than by the presence of VOCs derived from a single pathway within a species. This observation suggests a selection for complex profiles rather than for a specific pathway.

By plotting the principal component scores of each species (**Figure 6**), we found that the scores for each species along the first and second principal component (PC1 and PC2) axis most display a considerable spread. *A. braun-blanquetii* and *A. majus* Line Sippe 50, for example, have a large variation in scores along PC1 and PC2, reflecting findings from the cluster analysis. Indeed, developmental stages for these species did not cluster as tightly as for other species.

DISCUSSION

In the present study, we have determined the phenotypic space of scent profiles in eight wild species of *Antirrhinum* and two laboratory inbred lines. The species used in the present study are found in very distant regions of the Iberian Peninsula and have very different ecological niches. Our data show that the complexity of scent profiles in the *Antirrhinum* genus is remarkable with at least 63 different compounds previously identified, and an additional set of nine that may require further studies to verify their presence in plants. The number of independent major VOCs found is similar to most species described. Species have been identified with as little as one compound emitted by *Nicotiana africana* to 35 in *N. bonariensis* during the night (Raguso et al., 2006). Other species such as

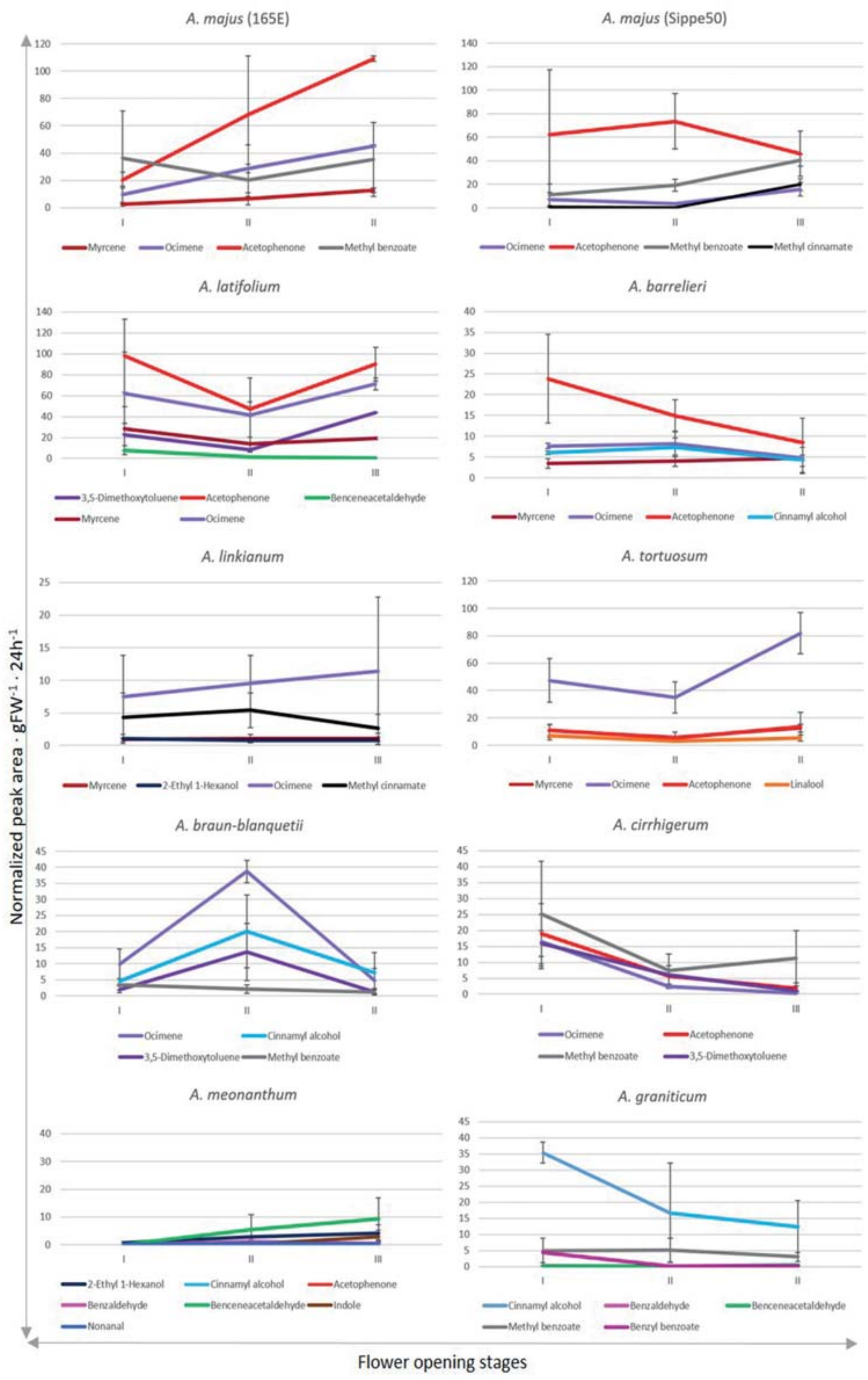

FIGURE 4 | Changes in emission of selected VOCs after anthesis. Stages of development described as stage I, II, and III correspond to 1–2, 3–4, and 5–6 days after anthesis. Quantities are reflected as total peak integrated areas normalized to naphthalene/g 24 h^{-1}. Error bars correspond to standard error. The VOCs represented here have an emission above 5% of the total amount of emitted VOCs.

Petunia (Kondo et al., 2007) have a range of scent components between 10 and 21, similar to the one found in the current study. The diversity of compounds produced in *Antirrhinum* is large but 500 VOCs have been described in studies in roses demonstrating the possibility of being a highly complex trait (Knudsen et al., 2006).

The most common volatiles found in between 71 and 52% of all plant families are, in decreasing order, limonene, ocimene,

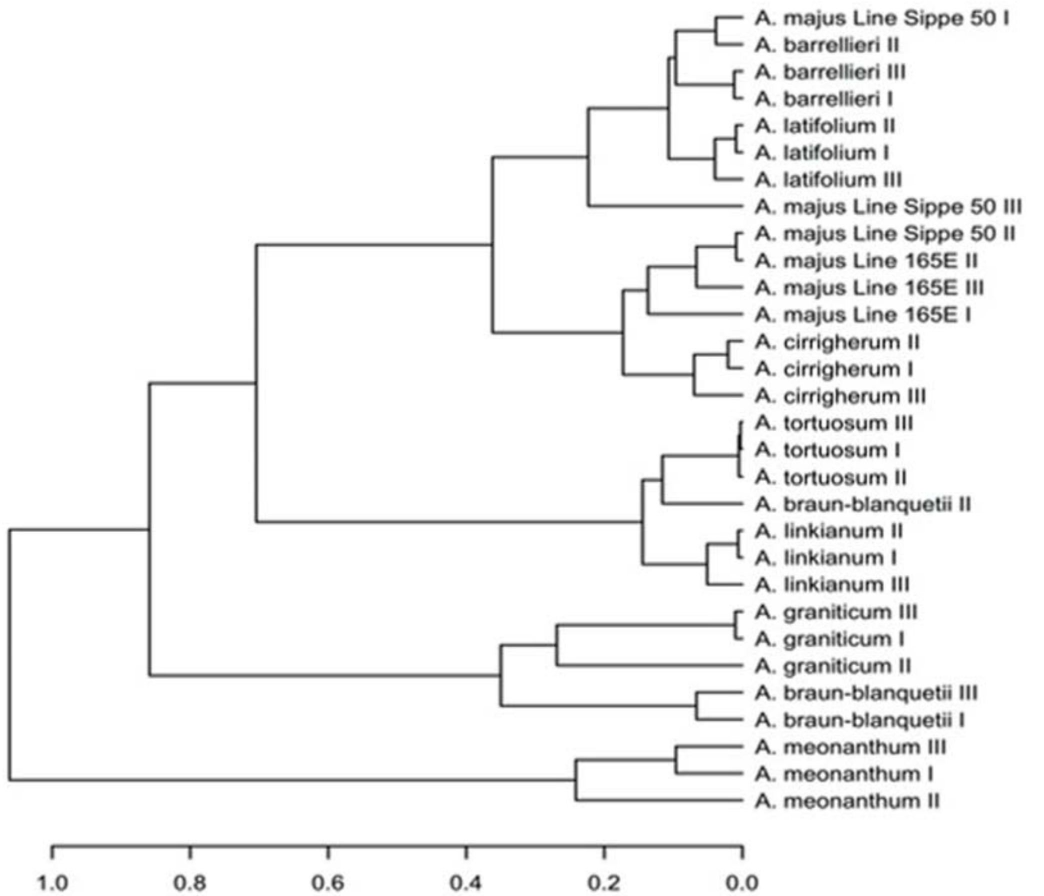

FIGURE 5 | Cluster analysis based on scent profiles of the different *Antirrhinum* species sampled at different flower developmental stages.

TABLE 4 | Principal component loadings for the four principal components explaining more than 80% of the variance.

Compound	PC1 (58.32%)	PC2 (10.90%)	PC3 (7.20%)	PC4 (5.89%)
Benzaldehyde	−0.158	−0.245	−0.118	0.064
Benzeneacetaldehyde	−0.113	−0.227	−0.156	0.079
Methyl benzoate	−0.328	−0.150	0.089	**−0.659**
Cinnamaldehyde	−0.043	−0.151	0.153	−0.062
Cinnamyl alcohol	−0.226	**−0.469**	**0.589**	−0.081
Methyl cinnamate	−0.111	−0.147	−0.170	0.109
Acetophenone	**−0.487**	0.316	**−0.395**	−0.396
3,5-Dimethoxytoluene	−0.271	−0.042	0.147	0.060
Indole	−0.090	−0.284	−0.099	0.061
1-Hexanol, 2-ethyl-	−0.191	**−0.367**	−0.381	0.214
Nonanal	−0.059	−0.169	−0.176	0.063
a,β-Ocimene	**−0.537**	0.277	0.235	**0.423**
β-Myrcene	−0.310	0.252	0.034	0.187
α-Farnesene	−0.061	0.003	−0.065	0.155

The variance explained by each component is indicated in parentheses. Only compounds with a loading ≥| 0.15| in at least one component are shown. Loadings with highest correlation to individual components are in boldface.

myrcene, linalool alpha-pinene, benzaldehyde, ß-pinene, methyl 2-hydroxybenzoate also known as methyl salicylate, benzyl alcohol, 2-phenyl ethanol, caryophyllene, and 6-methyl-5-hepten-2-one (Knudsen et al., 2006). The compounds found in most *Antirrhinum* species thus fall within the major VOCs found in flowering plants. The main VOCs found in all the *Antirrhinum* accessions analyzed were benzaldehyde, acetophenone ocimene and the fatty acid derivative 2-ethyl 1-hexanol indicating a common set of VOCs in the species analyzed. Highly ubiquitous compound such as benzyl alcohol was clearly forming a separate

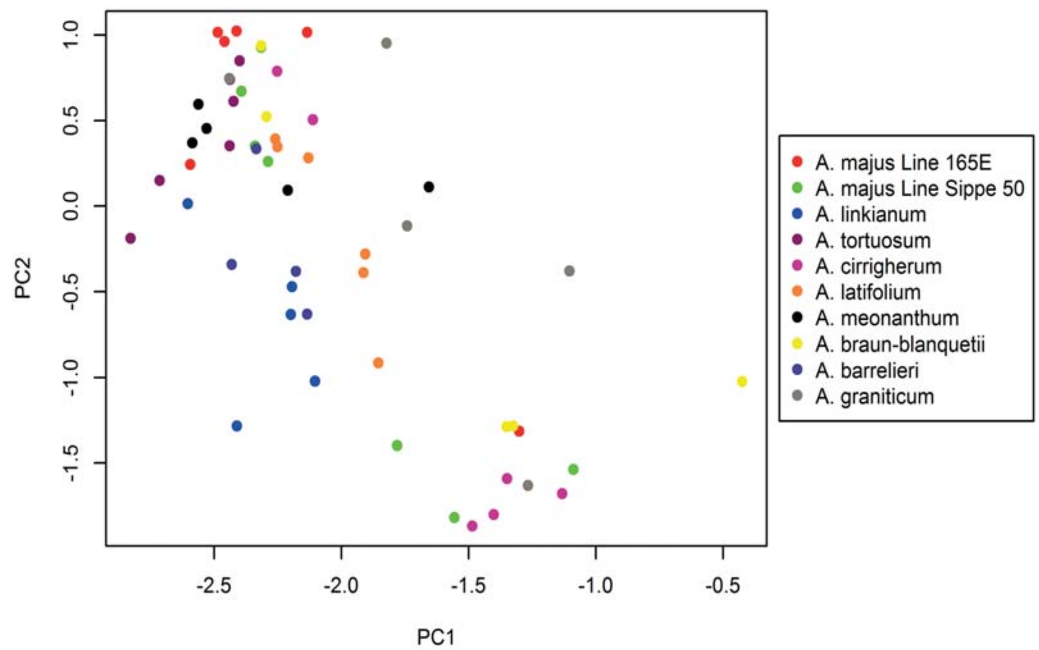

FIGURE 6 | Principal component analysis (PCA) of *Antirrhinum* species based on emitted volatiles. The two axes represent principal components 1 and 2, which explain 58.32% and 10.90% of the total variance, respectively.

group of accessions that either did or did not produce this specific compound (**Figure 2**). The only common scent compound we did not find was caryophyllene (Knudsen et al., 2006), and others such as the commonly found limonene, or alpha and beta pinene were detected only in trace amounts. Other compounds found less often but still generally found in plants included indole. Altogether the major VOCs found in *Antirrhinum* are a good representation of the different biosynthetic pathways found for scent VOCs in the plant kingdom. This is in sharp contrast to well established models such as *Arabidopsis* that produces sesquiterpenes as major VOCs (Tholl et al., 2005), or Petunia producing mainly phenylpropanoids (Hoballah et al., 2007; Bombarely et al., 2016).

The scent profile of flowers of a specific plant can change in response to the physiological stage of the flower, including flower age (Pichersky et al., 1994; Dudareva et al., 1998), pollination status the circadian rhythm or temperature (Sagae et al., 2008; Cna'ani et al., 2014). We found that all the species analyzed except two, *A. meonanthum* and *A. latifolium,* displayed an increase in emission followed by a decrease after 5–6 days after anthesis. Moreover, *A. majus* Sippe 50 and *A. graniticum* also had a major VOC showing a trend increasing towards the end of the flower lifespan. As the compounds showing this trend were very diverse including acetophenone, ocimene, 2-ethyl hexanol, indole, cinnamyl alcohol, and methyl cinnamate, we cannot conclude that it is a single pathway that is differentially regulated during flower aging. Our results indicate that there must be a common mechanism of control involved in the quantitative control of scent emission linked to flower aging, and this mechanism is subject to changes as found for individual components that differed in the emission kinetics.

The diversity among the major compounds was strong enough to allow a phylogenetic reconstruction. There are several phylogenies described for the genus *Antirrhinum*, including reconstructions based on chloroplast genes such as combined *psbA-trnH/trnT-trnL/ trnK-matK/trnS-trnG* sequences (Carrio et al., 2010), *trnS-trnG/trnK-matK* (Liberal et al., 2014) statistical parsimony networks of plastid haplotypes *trnS-trnG* and *trnK-matK* (Vargas et al., 2009), the nuclear *CYCLOIDEA* gene (Gübitz et al., 2003), and AFLP nuclear markers (Wilson and Hudson, 2011). All the aforementioned studies show *A. meonanthum* and *A. braun-blanquetii* are on a single clade while the rest of the species analyzed in the current study cluster together. Our data, show clustering of *A. menonanthum* and *A. braun-blanquetii*, while the other species form a different clade. Thus we can conclude that the complex scent profiles and both chloroplast and nuclear markers show a similar separation. A current hypothesis is that a multilocus under selection pressure maybe responsible for the complex phylogeny of *Antirrhinum* (Wilson and Hudson, 2011). Indeed the major local pollinators have been analyzed for three different *Antirrhinum* species and they are different (Vargas et al., 2010). Amongst the species studied, two are present in our work, i.e., *A. braun-blanquetii* and *A. graniticum*. However, we do not have evidence about a co-evolution or selection of the different scent profiles found in the different species and local pollinators, and they could be the result of a combination of selection and genetic drift. Variation between the different species is not based on single pathways but appears to occur at the aroma level, i.e., at the level of combination of components. As the levels of monoterpenes in *A. meonanthum* and *A. graniticum* are nearly absent, it remains to be determined if these changes are the result of single mutations affecting regulatory elements or key enzymes in the biosynthesis pathway.

The number and type of VOCs found in the *Antirrhinum* species analyzed indicated that there are several biosynthetic pathways that in parallel give rise to the scent blends identified. An important question raised is if the different profiles identified are the result of differences in biosynthetic pathways or rather result from the combination of components. As compounds belonging to a single pathway maybe correlated they would obscure statistics. Our data show that this is not the case. The first two compounds accounting for 58% of the variance correspond to the benzenoid acetophenone and the terpenoid ocimene, indicating that the major compounds do not belong to single pathways. This was corroborated with the other compounds that showed significant effects shared by cinnamyl alcohol, a benzenoid and 1-hexanol-2-ethyl, a fatty acid derivative. Altogether the PCA analysis indicates that the different scent profiles identified are not the result of changes in regulatory pathways or changes in one specific type of scent compound, suggesting a scent structure based on blends in *Antirrhinum*. This is not always the case as scent profiles with major components belonging to distinct pathways have been identified (Majetic et al., 2007). Our results do not exclude the possibility of finding other species where changes in regulatory genes or key enzymes will cause changes in complete VOC biosynthesis pathways. Indeed the only *Antirrhinum* wild species described so far *A. majus. Ssp pseudomajus* and *A. majus ssp striatum* differ in the emission of three benzenoids (Suchet et al., 2010), indicating a complex scenario in terms of scent profiles and differences between species.

Our data show that in general the *Antirrhinum* genus tends to have a robust scent profile. The fact that *A. braun-blanquetii* and *A. majus* Sippe 50 display modified scent profiles with aging indicates a genetic component establishing the complete scent profile. In this case it is not the effect of a single master activator as scent was produced by both species. As a pathway of regulatory genes plays a key role in control of scent production in Petunia (Klahre et al., 2011; Van Moerkercke et al., 2011; Spitzer-Rimon et al., 2012), activation of floral scent production in *Antirrhinum* at anthesis may be controlled by several non-redundant genes. Our results also suggest that the use of scent profiles for phylogenetic analysis may require sampling at different ages or developmental stages in order to define profiles that may or may not resolve distances. The richness of volatiles and the marked differences between the different species open the possibility to study the genetic structure of scent as a trait, and its use in evolutionary studies. The robustness of scent profiles may be seen as a signature and it may help in creating fidelity to pollinators.

AUTHOR CONTRIBUTIONS

JW, JM, ND, and ME-C designed experiments; JW, obtained data; JW, JM, VR-H, and ME-C analyzed data; JW, JM, VR-H, ND, and ME-C wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01903/full#supplementary-material

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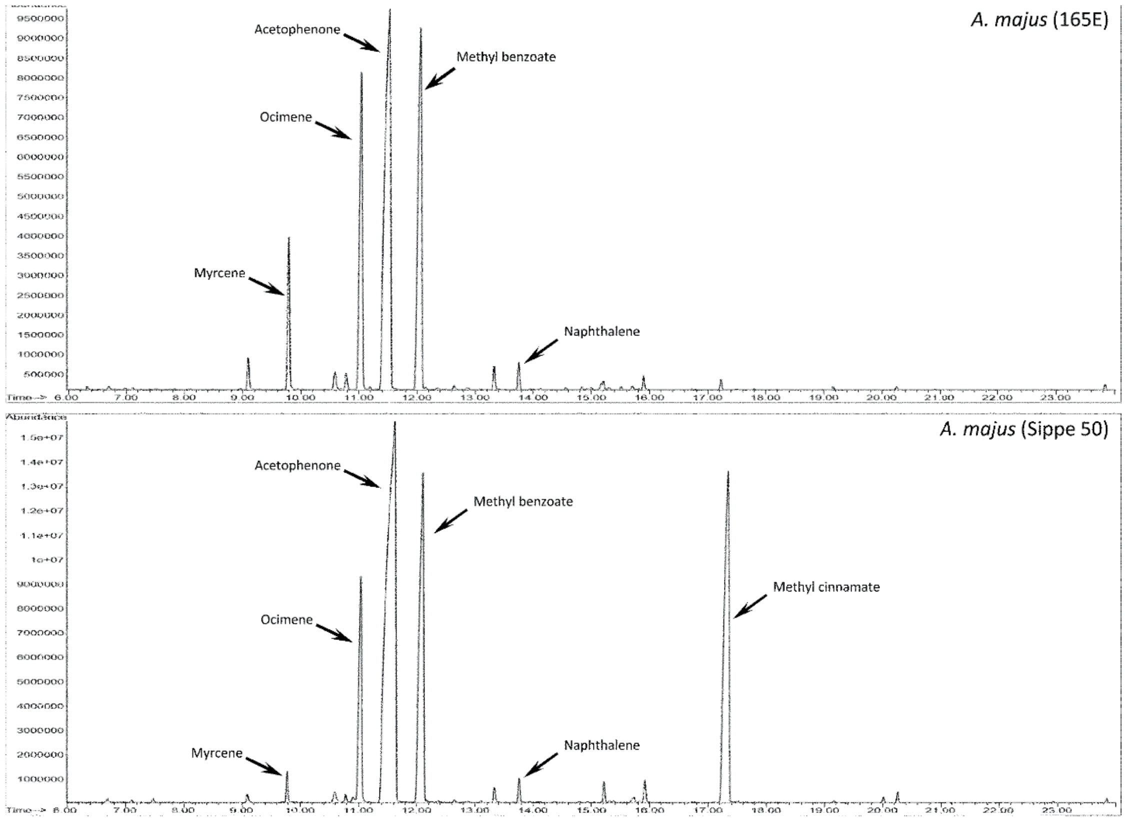
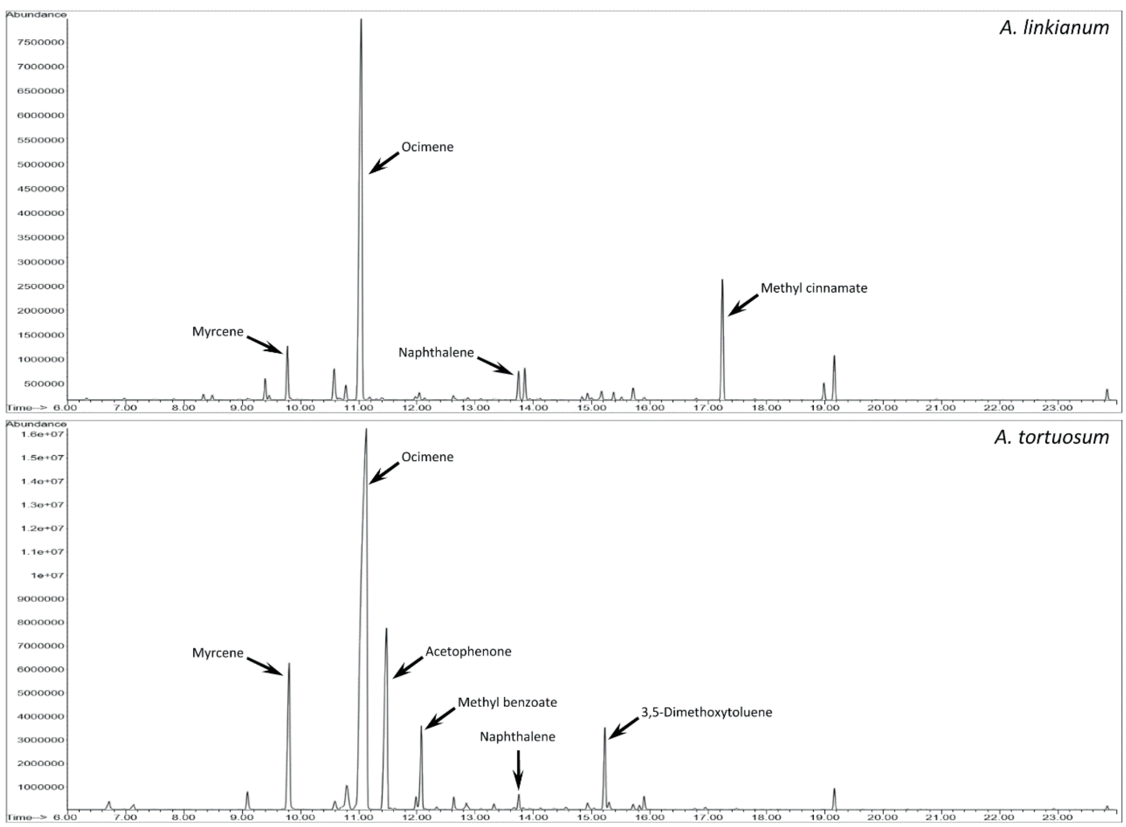
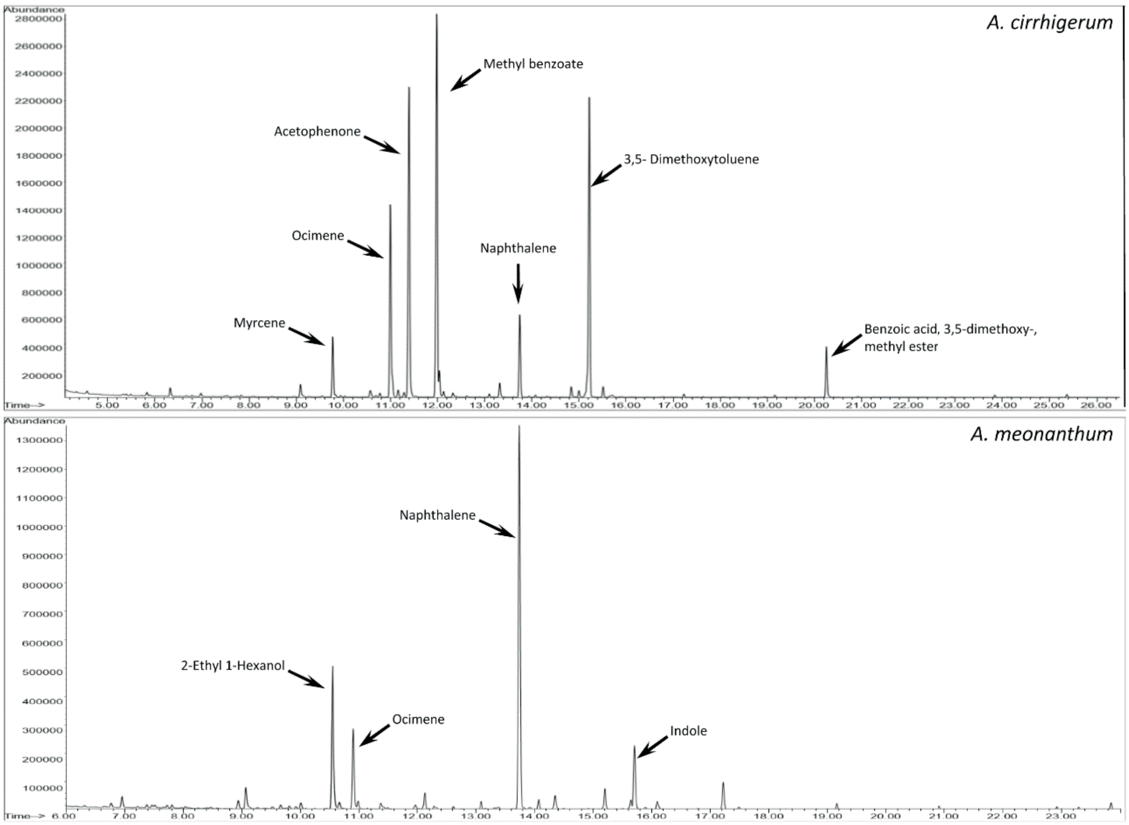
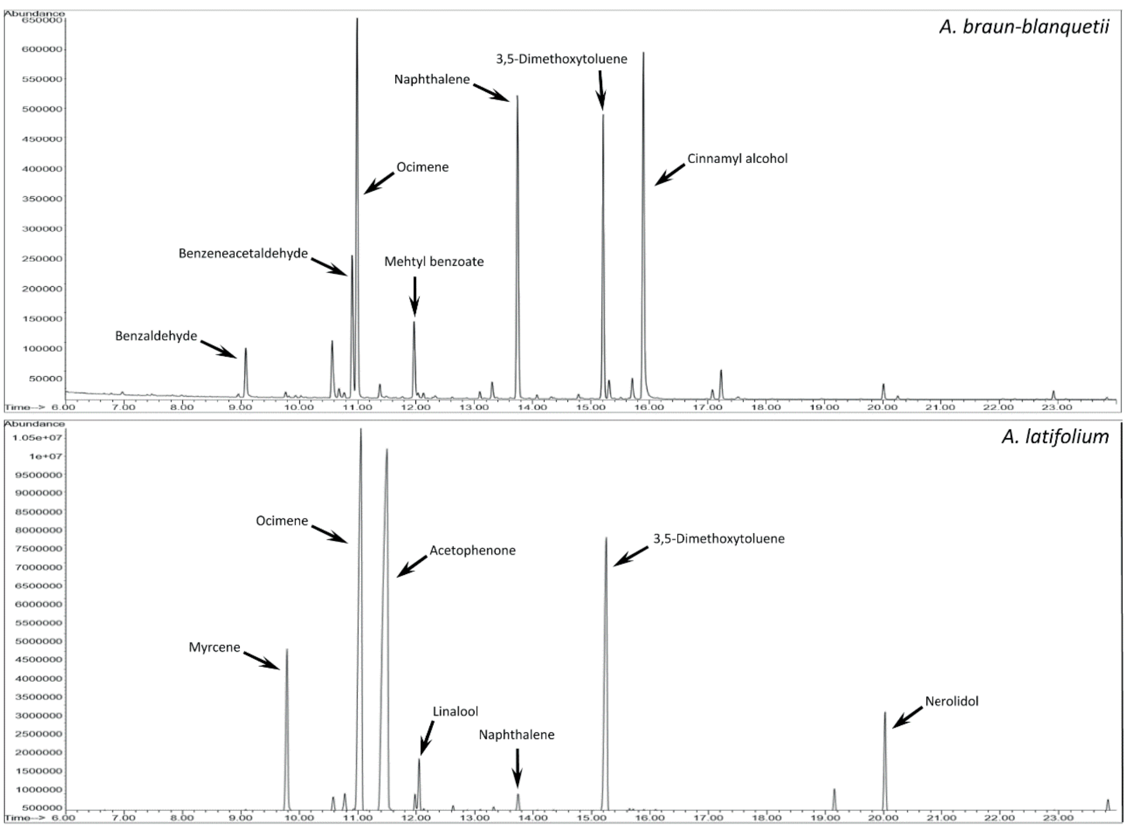
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material






A. cirrhigerum
Methyl benzoate
Acetophenone
3,5- Dimethoxytoluene
Ocimene
Naphthalene
Myrcene
Benzoic acid, 3,5-dimethoxy-, methyl ester
A. meonanthum
Naphthalene
2-Ethyl 1-Hexanol
Ocimene
Indole
Abundance
Time-->


A. braun-blanquetii
3,5-Dimethoxytoluene
Naphthalene
Cinnamyl alcohol
Ocimene
Benzeneacetaldehyde
Mehtyl benzoate
Benzaldehyde
A. latifolium
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Nerolidol
Abundance
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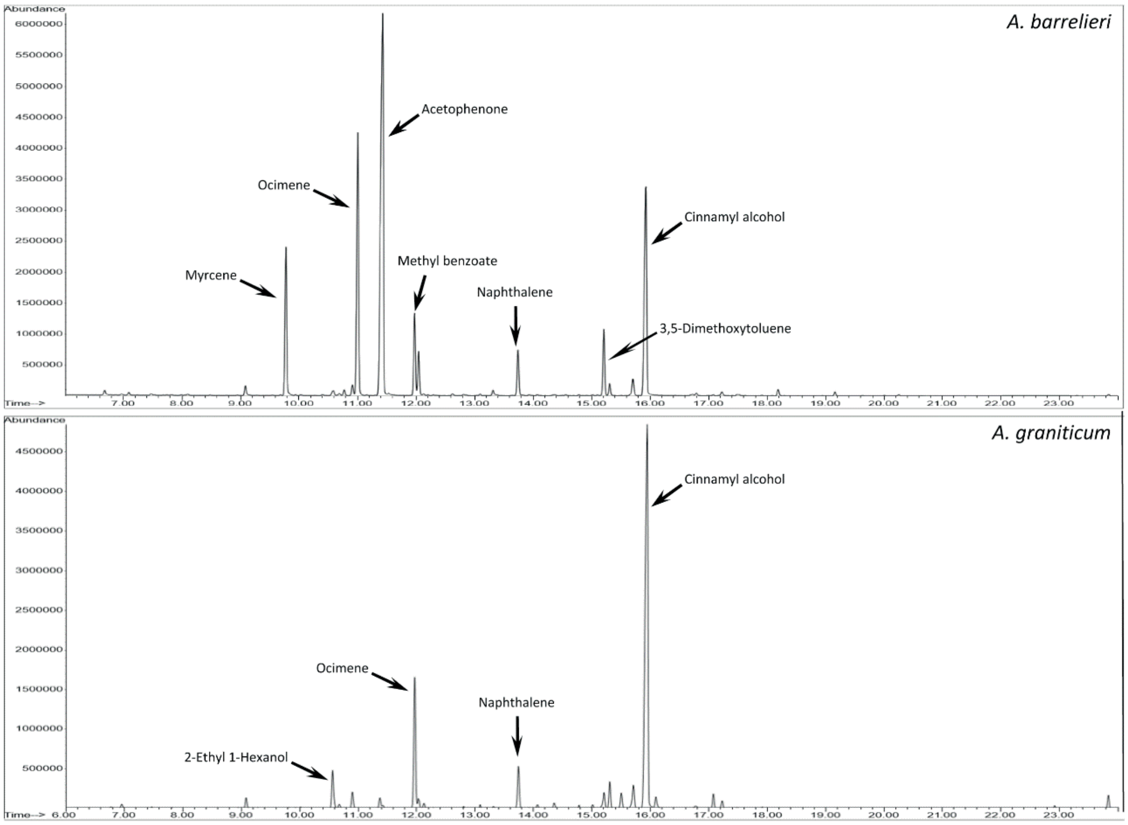

Figure S1. Gas chromatograms of volatiles of *Antirrhinum*. Chromatograms correspond to stage III for all species. Peak areas are representative of emitted quantities, and the X axis corresponds to retention times. Major peaks are depicted and naphthalene is also shown as internal standard. The naphthalene peak corresponds to 100 nanograms.

Table S1. Percentage of major volatile compounds in *Antirrhinum majus* laboratory inbred lines and wild species (*A. linkianum, A. tortuosum, A. cirrhigerum, A. meonanthum, A. braun-blanquetii, A. latifolium, A. barrelieri* and *A. graniticum*). Percentage is based on volatile collection from three radomly chosen flowers at flower opening stages I = day 1 -2; II = day 3 -4; III day 5 -6. Volatiles that account for ≥1% of total compounds in at least two samples of one species are listed. Information of main flower colour included for each species.

	***A. majus* line 165E**			***A. majus* line Sippe 50**			***A. linkianum***			***A. tortuosum***			***A. cirrhigerum***		
Flower colour	pink-white			magenta			magenta			yellow-white			magenta		
Flower opening stage	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Benzenoid-Aldehydes															
Benzaldehyde	3.0	2.8	1.3	0.1	0.5	0.2	3.1	0.5		1.6	0.8	0.9	0.6	1.1	0.3
Benzenoid - Ketones															
Acetophenone	20.3	33.5	43.9	70.8	39.0	46.5		0.2		12.5	9.5	14.5	20.3	23.8	33.5
1-(2-hydroxyphenyl) ethanone	0.6	0.7	0.9	1.1		0.4				0.3			1.6	1.1	0.6
Benzenoid - Esters															
Methy benzoate	55.7	41.7	25.6	12.3	40.4	19.4	1.5	3.0	0.2	1.1	0.2	0.6	31.3	28.0	21.0
Benzenoid - Ethers															
3.5-Dimethoxytoluene	0.9	0.2	0.3	0.1						2.1	1.4	4.0	17.3	22.7	18.9
Isoprenoids-Monoterpenes															
Myrcene	2.8	2.7	6.0	1.9	2.5	0.7	5.4	4.3	4.9	10.5	10.2	9.3		4.0	4.46
Ocimene	12.5	14.5	18.3	8.1	10.9	9.0	23.5	44.1	59.3	56.9	65.3	58.6	16.8	14.0	17.0
Linalool								0.3	0.7	7	5.7	4.6		1.4	
Isoprenoids-Sesquiterpenes															
α-Farnesene	0.3	0.1	0.1	0.5	0.3		1.8	2.4	4	1.5		0.9	0.2		
Phenylpropanoids -Esters															
Methyl cinnamate	0.5	0.2	0.4	0.1	0.2	21.4	13.7	22.7	12.4				0.5	0.2	1.6
Phenylpropanoids - Alcohols															
Cinnamyl alcohol	0.3		0.5	1.5	1.0	0.6	1.5	2.6	0.3	0.2	1.5	0.7			
Fatty acid derivatives - Alcohols															
2-Ethyl 1-Hexanol		0.5	0.9	0.5	1.0	0.4	20.5	3.8	3.6	2.2	0.3	0.6	2.2	0.6	0.8

	A. meonanthum			*A. braun-blanquetii*			*A. latifolium*			*A. barrelieri*			*A. graniticum*		
Flower color	yellow			yellow			white			magenta			white-pink		
Flower opening stage	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Benzenoid-Aldehydes															
Benzeneaceta dehyde	9.9	2.7		0.1	0.7	0.1	0.5	6.3		0.6	0.3	0.7	2.6	6.9	1.6
Benza dehyde	8.2	10.0	3.0	2.0	1.8	1.3	0.7	2.0	0.1	0.6	0.2	0.7	2.6	6.9	1.6
Benzenoid - Ketones															
Acetophenone	2.5	8.7	5.0	0.3		0.2	49.0	21.4	32.8	33.5	57.9	38.1	1.0		1.0
Benzenoid - Esters															
Methy benzoate		2.3	1.1	1.9	4.7	6.6	0.7	1.1	1.8	0.9	1.5	5.0	5.4	36.5	13
Benzy Benzoate	4.2	1.0			0.25		0.1	0.8					0.6	9.4	0.3
Benzenoid - Ethers															
3.5-D methoxyto uene		3.7	1.8	11.6	9.1	5.8	8.5	22.7	19.3	2.0	1.0	4.1	0.5		1.8
Benzenoids - Benzenes															
p-Xy ene	0.2	0.4		4.0	5.3			0.1	0.1				0.5		
1.4-D methoxybenzene				1.0	1.1	1.0									
Benzenoids - Alcohols															
Benzy A coho	3.0	5.3	1.9	0.5	0.9	0.5		1.0		0.2			1.2	2.7	0.5
Isoprenoids-Monoterpenes															
β-Myrcene				3.2	2.8	0.1	9.9	18.0	8.0	5.8	7.8	9.4			
β-Oc mene (Z)			0.3	50.1	44.0	25.6	24.8	14.8	28.96	17.9	13.8	19.9	1.2		
L na oo				0.8		0.5	1.6	1.3	2.2	1.5	2.2		1.8		1.1
Isoprenoids-Sesquiterpenes															
a-Farnesene	2.2	1.2			0.1		0.3	0.1	0.4	0.3	1.0	0.2			
Nero do				3.0	3.9	2.7	1.8		3.7						
Phenylpropanoids - Alcohols															
C nnamy a coho		8.0		19.0	23.6	40.1				14.2	10.8	17.4	65.2	32.5	60.7
Phenylpropanoids - Aldehydes															
C nnamy a dehyde		1.0		1.2	1.6	1.9				1.1	0.6	0.8	2.7	1.0	2.7
Fatty acid derivatives - Alcohols															
2-ethyl 1-Hexanol	45.0	16.4	25.0	0.9	0.1	2.1			0.5	1.2	0.4		2.8		4.1
Fatty acid derivatives - Aldehydes															
Decana	2.4	3.9	1.2		0.1	0.1	0.1			0.2			0.4	0.8	0.3

Nonana	2.9	6.8	2.2	0.1	0.2		0.2	0.4	0.1	0.4			0.7	1.1	0.5
Fatty acid derivatives - Ketones															
Hexahydrofarnesy acetone	2.9	2.1	1.5	0.3	0.2	0.5	0.3	2.0	0.4		0.2		1.0		1.2
Amines a other nitrogen containing compounds															
Indo e	5.6	7.4	18.1	1.2	1.0	2.1			0.1	0.2	1.1	1.2	1.9	1.2	3.0

Chapter II

Genetic Analysis of Natural Variation in Antirrhinum Scent Profiles Identifies BENZOIC ACID CARBOXYMETHYL TRANSFERASE as the Major Locus Controlling Methyl Benzoate Synthesis

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Genetic Analysis of Natural Variation in *Antirrhinum* Scent Profiles Identifies BENZOIC ACID CARBOXYMETHYL TRANSFERASE As the Major *Locus* Controlling Methyl Benzoate Synthesis

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The *Antirrhinum* genus has a considerable complexity in the scent profiles produced by different species. We have analyzed the genetic differences between *A. majus* and *A. linkianum*, two species divergent in the emission of methyl benzoate, methyl cinnamate, acetophenone, and ocimene. The genetic analysis showed that all compounds segregated in a Mendelian fashion attributable to one or two *loci* with simple or epistatic interactions. Several lines lacked methyl benzoate, a major Volatile Organic Compound emitted by *A. majus* but missing in *A. linkianum*. Using a candidate gene approach, we found that the *BENZOIC ACID CARBOXYMETHYL TRANSFERASE* from *A. linkianum* appeared to be a null allele as we could not detect mRNA expression. The coding region did not show significant differences that could explain the loss of expression. The intron-exon boundaries was also conserved indicating that there is no alternative splicing in *A. linkianum* as compared to *A. majus*. However, it showed multiple polymorphisms in the 5′ promoter region including two insertions, one harboring an *IDLE* MITE transposon with additional sequences with high homology to the *PLENA locus* and a second one with somewhat lower homology to the regulatory region of the *VENOSA locus*. It also had a 778 bp deletion as compared to the *A. majus BAMT* promoter region. Our results show that the differences in scent emission between *A. majus* and *A. linkianum* may be traced back to single genes involved in discrete biosynthetic reactions such as benzoic acid methylation. Thus, natural variation of this complex trait maybe the result of combinations of wild type, and loss of function alleles in different genes involved in discrete VOCs biosynthesis. Furthermore, the presence of active transposable elements in the genus may account for rapid evolution and instability, raising the possibility of adaptation to local pollinators.

Keywords: recombinant inbred lines, floral scent, transposable element, *IDLE* MITE, methyl benzoate, acetophenone, β-ocimene, methyl cinnamate

INTRODUCTION

The study of natural variation in natural populations has a great potential for identifying the genetic structure of complex traits. Studies in plants using natural populations have helped to identify a large number of genes involved in different traits such as flowering time, plant architecture, or biomass production (Alonso-Blanco et al., 2009). One of the major traits in plants is the production of secondary metabolites as it can be considered an interface of interaction with living organisms including bacteria, fungi, other plants, and of course animals. The emission of scent by flowers is considered a key trait to attract pollinators and repel potential pests (Schiestl, 2010).

One of the characteristics of floral scent is the complexity in terms of the number of independent Volatile Organic Compounds (VOCs). Some plants such as roses have over 500 VOCs (Spiller et al., 2010), and there are over 1700 compounds identified in floral scent (Knudsen et al., 2006). The genetic studies on scent and volatiles have been an important part of plant biotechnology as the major compounds involved in flavor and aroma are VOCs. As a result, studies in crops such as tomato, peach, rice or strawberry are well-developed (Zorrilla-Fontanesi et al., 2012; Sánchez et al., 2013; Rambla et al., 2014; Golestan Hashemi et al., 2015). The genetics of scent emission and its control has been studied in a variety of plants with different outcomes. Several components of the complex scent profiles of roses have been resolved to single Mendelian *loci* of one or two genes involved in the synthesis of single VOCs such as nerol, neryl acetate, and geranyl acetate (Spiller et al., 2010). A similar situation has been identified in *Mimulus*. The differences in pollinator choice in bumblebee-pollinated *Mimulus lewisii* and hummingbird-pollinated *M. cardinalis* are the result of changes in three volatiles: D-limonene, β-myrcene, and E-β-ocimene (Byers et al., 2014a). The genetic differences lie in two *loci* coding for a *LIMONENE-MYRCENE SYNTHASE* and an *OCIMENE SYNTHASE* (Byers et al., 2014b). A different situation has been described in Petunia. The Petunia genome has a multi *locus* island involved in the control of scent emission, floral visible color, UV absorption, pistil length, and stamen length (Hermann et al., 2013). Differences between the scented *Petunia axillaris* and the unscented *P. exserta* lie on two *loci*. One is a single *MYB* gene allelic to *ODORANT1* (*ODO1*) involved in the activation of phenylpropanoids synthesis (Klahre et al., 2011), pointing to a possible evolution at the regulatory level. There is emerging evidence from Petunia and *Antirrhinum* that scent and floral size may share some corregulators such as *ENHANCER OF BENZENOID II* in Petunia, involved in scent emission, flower opening, and anthesis (Colquhoun et al., 2011; Van Moerkercke et al., 2011). In *Antirrhinum* the gene *COMPACTA* is involved in maintenance of B function affecting petal size and scent emission (Manchado-Rojo et al., 2012). A study of narrow sense heritability in *Brassica rapa* has shown that although scent profiles between species differ in many cases in a number of independent VOCs, they may have coregulation between them and with other morphological traits or flowering time (Zu et al., 2016). The genes involved in single compound biosynthesis together with regulatory *loci* with several genetic functions indicates that the natural variation of scent emission may identify both regulatory *loci* as in Petunia and structural genes involved in discrete VOCs biosynthesis, or form part of larger pathways, affecting downstream products.

The systematic identification of enzymes responsible for the biosynthesis of many volatiles and secondary metabolites has followed a standard protocol. Coding regions have been expressed in a heterologous system such as bacteria, yeast or plant cells, and the enzyme activity has allowed the identification of the corresponding basic biochemical properties such as Km for different substrates. Such studies have determined enzyme activities involved in biosynthesis of alkaloids, phenylpropanoids, benzenoids etc. (Murfitt et al., 2000; Collu et al., 2001; Dudareva et al., 2003). However, the identification of genetic variation and the corresponding allelic differences allowing unequivocal annotation of the different coding genes in plants is lagging behind. This is especially important as some enzymes have been found to be able to produce more than one product *in vitro* and may act on different substrates *in vivo*.

The genus *Antirrhinum* comprises roughly 28 species with a center of origin in the Iberian Peninsula (Vargas et al., 2009). Work in *Antirrhinum* has shown that methyl benzoate, myrcene, and nerolidol are produced in a circadian fashion (Kolosova et al., 2001a; Dudareva et al., 2005). The complexity of scent components is reflected in the identification of at least 120 VOCs previously described in plants (Weiss et al., 2016b). In this work, we have performed a genetic analysis of scent emission spanning three generations following a cross of *A. majus* and *A. linkianum.* Both species differ in the production of four VOCS: methyl benzoate, β-ocimene, methyl cinnamate, and acetophenone. These compounds displayed Mendelian segregations typical for a single gene or two *loci* in the F2 population. We identified a loss of function allele of *BENZOIC ACID CARBOXYMETHYL TRANSFERASE* (*BAMT*), a gene involved in methyl benzoate synthesis in higher plants. The null allele is the result of a genomic insertion in the promoter region that was likely mediated by an *IDLE* MITE transposable element (Cartolano et al., 2007) in conjunction with additional genomic rearrangements including a second insertion of genomic sequences with similarity to the *VENOSA locus* (Schwinn et al., 2006). The underlying activity of transposable elements may represent a mechanism for the rapid evolution of scent profiles by promoting genomic rearrangements in key VOC biosynthetic enzymes or their regulatory elements.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Plants of *Antirrhinum majus* and *A. linkianum* were grown as described previously as a single plant per pot allowing maximum number of flowers to be produced (Weiss et al., 2016a). We performed a cross between *A. majus* line 165E and *A. linkianum* to obtain a recombinant inbred line.

VOC Collection

Plants belonging to the F2 or F3 segregating population were sampled once and in those cases where little or no scent was obtained a second resampling was performed. Flowers were incubated in a 25 ml glass beaker containing 4 ml of 5% glucose. The pedicel was in contact with the solution. The glass beakers were placed inside 1 l glass desiccators under a regime of 12:12 light dark and 23–18°C conditions. Samples were taken for 24 h periods. Flowers were weighted before and after the scent collection. Quantification of scent emission was based on flower total emission.

Gas-Chromatography Mass Spectrometry

Trapped floral volatiles were analyzed by gas chromatography–mass spectrometry (GC-MS) as described (Manchado-Rojo et al., 2012). Data analysis and volatile identification was performed with the MSD ChemStation (Agilent Technologies) software.

For semi-quantifying the main VOC compounds of the RILs (methyl benzoate, methyl cinnamate, acetophenone, and β-ocimene) we used standard solutions (Sigma-Aldrich products codes: 18344, 96410, 42163, W353901) diluted with methanol. The concentration of β-ocimene ranged from 25 to 1250 ppm, whereas the concentration of the rest of compounds ranged from 50 to 2500 ppm. An injection volume of 0.5 μl was applied directly to a Twister^{TM}. The standards were directly injected using a split/splitless injector (Agilent Technologies). Calibration curves were calculated by Chemstation (methyl benzoate: $1.181*10^7x - 1.009*10^5$, $r^2 = 0.999$; methyl cinnamate: $1.762*10^7x - 5.245*10^5$, $r^2 = 1$; acetophenone: $1.052*10^7x - 2.693*10^5$, $r^2 = 0.999$; β-ocimene (Z): $8.318*10^6x - 1.397*10^5$, $r^2 = 0.999$). The corresponding calibration curves were used to quantify the major compounds segregating. Total amounts are given in $\mu g \cdot flower^{-1} \cdot 24\ h^{-1}$ and in $\mu g \cdot$fresh weight (fw) $^{-1} \cdot 24\,h^{-1}$.

Cloning of *A. linkianum BAMT*

We obtained sequence information of *Antirrhinum majus* and developed PCR primers to amplify the genomic region corresponding to BAMT (AF198492.1). We amplified the complete coding region and 2.1 kb corresponding to the 5′ region upstream of the coding region from *A. majus 165E* and *A. linkianum* (Table S1) using TAKARA PrimeStar GXL TAQ polymerase. The amplified DNA fragments were T/A cloned in pGEMTEasy according to the manual and grown in DH10B *E. coli*. DNA sequence was determined by Sanger using standard primers for pGEMTeasy. The accession numbers of the sequences are KU512977 *A. majus BAMT* and KU512978 for *A. linkianum BAMT*.

qPCR

We analyzed the steady state accumulation of transcripts coding for *BAMT* by qPCR as described before (Delgado-Benarroch et al., 2009). Petals were sampled at T6 subjective time being T0 dawn, as it coincides with the highest *AmajusBAMT* expression (Kolosova et al., 2001a). Total RNA was extracted from fully developed petals using the RNAEasy Kit from Macherey and Nagel according to the instructions. Genes were amplified in a Stratagene Mx3000 qPCR machine (www.agilent.com), with sequence-specific primers (Table S1) synthesized by Invitrogen (www.invitrogen.com) using Takara SYBR-Green (www.thermofischer.com). We used the gene *UBIQUITIN CONJUGATING ENZYME2* (accession number AJ560266.1; Bey et al., 2004) as a control for normalization. The PCR program was performed with 45 cycles including a 10 min denaturation at 95° followed by 30 s at 56°, 45 s at 72°, and 30 s at 95°. We performed experiments with one biological replica and two technical replicas for each of the lines analyzed. The experiment was repeated twice including mRNA extractions with similar results.

RESULTS

Construction of Recombinant Inbred Lines

We had previously found that *Antirrhinum majus* 165E and *A. linkianum* differ in the emission of four main floral scent VOCs, methyl benzoate, methyl cinnamate, β-ocimene, and acetophenone (Weiss et al., 2016b). In order to identify the genetic components involved in the differential emission, we constructed an F2 from *A. majus* line 165E × *A. linkianum* (**Figure 1**). The *A. majus* line 165E has a strong activity

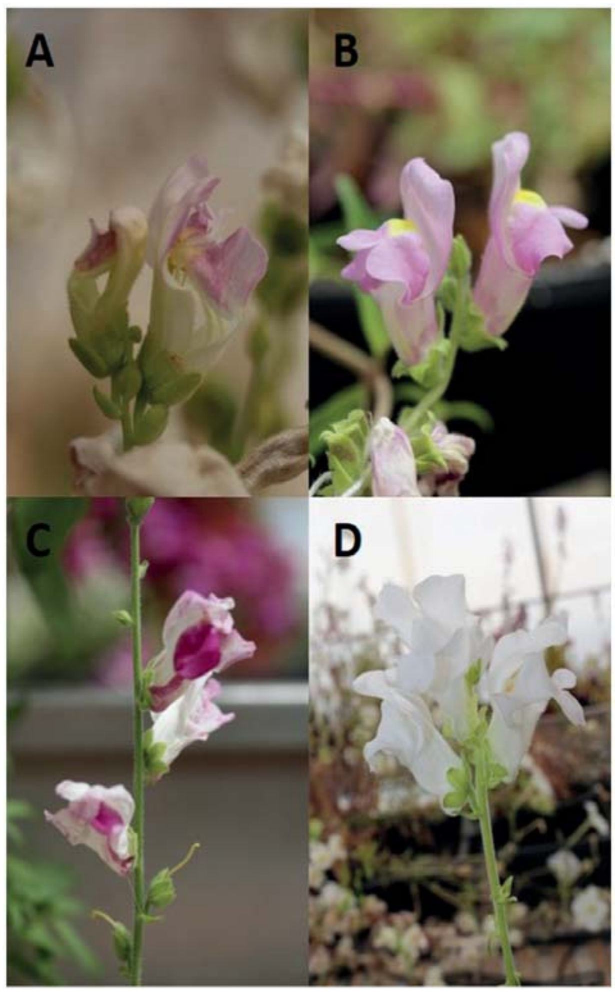

FIGURE 1 | Pictures of flowers of (A) *Antirrhinum majus* 165E **(B)** *A. linkianum* and two F2 siblings differing in color. **(C)** The transposon activity could be noted and **(D)** showing white flowers.

of transposable elements and is mutated in the *PALLIDA locus* coding dihydroflavonol-4-reductase (Martin et al., 1985). This enzyme catalyzes the reduction of dihydroquercetin to leucocyanidin, during the last steps in anthocyanin biosynthesis thus allowing the visual monitoring of transposon activity in the flowers.

We obtained an initial population of 174 F2 plants. We used a total of 110 F2 plants that flowered during a period of 5 months to analyze scent production and selfed the plants to obtain further inbred lines.

Genetic Analysis of Scent Profiles

We identified a number of individuals in the F2 that produced either low or close to undetectable levels of the four VOCs that are contrasting between the parental lines (**Table 1**). We analyzed the segregation of the compounds and found a range of methyl benzoate emission between 203.41 μg·flower^{-1}·24 h^{-1} from line 39 and down to 1.12 μg·flower^{-1}·24 h^{-1} from line 1 (**Figure 2**). The emission of β-ocimene ranged between 372.91 μg·flower^{-1}·24 h^{-1} from line 4 and 1.85 μg·flower^{-1}·24 h^{-1} from line 96. The emission of methyl cinnamate ranged between 48.07 μg·flower^{-1}·24 h^{-1} from line 102 and was 0.03 μg·flower^{-1}·24 h^{-1} from lines 16, 29, 31, 36, 43, 62, 86, 108, and 112. Finally, acetophenone emission ranged between 304.29 μg·flower^{-1}·24 h^{-1} from line 19 and down to 0.03 μg·flower^{-1}·24 h^{-1} from line 47.

We analyzed the data of the different scent components to identify a possible genetic model of segregation for each of the VOCS, considering the current knowledge at the biochemical level (**Figure 3**). The enzyme benzoic acid carboxymethyltransferase (BAMT) is the major enzyme involved in the synthesis of methyl benzoate (Dudareva et al., 2000; Murfitt et al., 2000; Effmert et al., 2005). The segregation analysis of plants producing high and low amounts of methyl benzoate complied to a model of a single gene with Mendelian 3:1 segregation (Chi square test $p = 0.582$), where the plants producing methyl benzoate were dominant (**Table 2**). This indicated that the allele of *A. linkianum BAMT* could be a major candidate gene involved in the synthesis of methyl benzoate. Furthermore *A. linkianum BAMT* should probably code for a loss of function allele.

The synthesis of β-ocimene in *A. majus* is performed by a specific β-ocimene synthase (Dudareva et al., 2003). The emission of β-ocimene followed a similar pattern as methyl benzoate but in contrast, the number of plants producing low or very low amounts was lower. In fact, we always found small amounts of β-ocimene indicating that a possible allele of *A. majus OCS* was a hypomorphic allele but not a complete null. We tested the hypothesis of a single gene segregating taking into account three different thresholds of emission. The most strict showed significant differences to a 3:1 Mendelian segregation ($p = 0.002953$; **Table 2**). A slightly less conservative cutoff at levels below 30 μg·flower^{-1}·24 h^{-1} did result in acceptable statistical fits ($p = 0.9.123$). Using the strict threshold below 10 μg·flower^{-1}·24 h^{-1}, we also found a possible model for a 13:3 segregation ($p = 0.1056$) where two genes would come into play (**Table 2**).

TABLE 1 | *A. majus* × *A. linkianum* (F2) emission of methyl benzoate, β-ocimene, methyl cinnamate, and acetophenone (μg·FLOWER^{-1}·24 h^{-1}).

Line	Methyl benzoate	β-ocimene	Methyl cinnamate	Acetophenone
1	1.12	37.35	0.20	0.74
2	42.91	103.67	4.26	10.06
3	3.69	175.77	0.56	186.78
4	53.32	372.91	15.48	260.36
5	37.56	92.09	0.30	32.01
6	15.72	11.64	0.39	2.61
7	13.17	97.24	0.46	91.23
8	6.98	79.04	15.20	2.98
9	1.22	94.81	3.52	0.26
10	126.67	100.64	1.08	11.95
11	123.12	35.91	0.43	75.31
12	30.62	32.59	0.86	9.43
13	40.25	31.57	23.67	303.03
15	80.92	57.42	7.04	66.73
16	1.39	7.99	0.03	0.49
18	54.50	51.17	0.29	27.95
19	17.13	186.93	2.19	304.29
21	5.25	97.26	0.52	0.15
22	44.19	113.57	0.08	11.94
23	47.14	96.41	0.10	88.92
24	37.50	29.06	0.74	29.53
25	11.08	5.62	0.75	40.70
27	0.56	82.47	3.10	0.21
29	14.30	53.98	0.03	48.07
31	43.09	29.12	0.03	14.26
32	1.05	65.38	1.25	0.77
33	11.92	71.76	0.12	35.70
34	1.14	11.89	5.25	0.88
35	38.17	3.28	1.09	74.16
36	2.01	1.95	0.03	2.38
37	94.29	210.09	13.99	3.70
38	2.24	76.58	0.09	1.51
39	203.41	325.85	4.04	12.29
40	42.63	36.71	1.50	88.72
41	15.59	32.86	2.25	19.46
42	67.66	32.76	1.11	24.65
43	66.69	143.26	0.03	0.56
44	37.82	72.15	0.15	160.01
47	146.77	284.77	35.47	0.03
48	74.83	67.91	45.13	25.83
51	162.06	236.10	2.42	246.65
52	11.64	3.89	0.08	7.11
53	58.37	20.86	0.09	53.34
56	3.75	5.25	22.78	1.65
57	43.21	69.50	1.77	19.54
58	60.24	7.48	1.76	79.76
60	26.40	4.73	0.19	20.00
62	3.71	28.65	0.03	25.30
64	2.52	75.33	2.50	0.85

(Continued)

TABLE 1 | Continued

Line	Methyl benzoate	β-ocimene	Methyl cinnamate	Acetophenone
65	4.55	78.02	0.23	14.31
66	74.04	105.48	2.38	125.28
67	2.87	97.26	0.27	4.94
68	54.99	138.41	38.11	69.35
69	21.47	14.26	30.28	2.87
71	12.28	73.29	0.99	19.83
72	16.3	13.58	4.75	96.63
73	1.22	21.24	19.07	2.27
74	5.17	240.55	14.42	1.1
75	67.83	298.36	11.63	122.75
76	87.3	126.57	1.55	19.56
78	1.13	91.79	0.81	93.54
79	86.43	81.75	2.05	64.87
80	46.83	2.55	3.77	55.66
83	41.98	199.34	5.08	3.2
85	43.1	130.71	0.1	133.19
86	52.21	103.41	0.03	42.49
87	77.98	199.87	1.75	289.0
88	97.71	220.32	9.82	7.0
89	53.87	74.45	2.43	9.15
90	16.0	84.57	0.37	21.21
91	39.11	62.58	0.84	15.83
93	33.63	199.62	5.55	3.92
95	26.36	23.56	0.14	11.41
96	12.46	1.85	16.44	2.67
97	68.79	52.54	16.42	172.94
98	5.8	51.46	20.51	3.69
99	139.65	51.17	21.95	166.28
100	60.08	22.76	6.12	17.67
101	19.24	28.21	0.8	1.4
102	64.38	31.43	48.07	6.94
103	89.71	146.16	0.2	7.2
104	13.37	3.32	0.33	1.1
105	19.74	63.48	25.82	3.51
106	28.71	90.44	12.03	1.74
107	1.76	54.66	6.81	3.63
108	42.9	79.91	0.03	266.72
109	168.38	148.08	0.1	373.39
110	64.48	128.9	3.33	18.46
111	95.91	22.79	0.6	35.11
112	88.53	118.99	0.03	75.56
113	61.93	41.29	3.41	108.44
114	2.88	166.18	0.12	1.52
115	66.17	85.87	0.08	71.67
116	1.92	2.97	0.37	2.29
117	195.26	82.87	0.2	211.54
118	36.98	4.44	0.66	33.23
119	25.78	30.85	0.76	44.79
120	84.16	99.84	0.13	61.18
121	29.72	104.68	10.87	126.79
122	69.82	87.67	10.87	123.69
123	21.13	78.98	17.6	0.49
124	60.34	154.03	0.08	184.3
125	64.47	193.11	9.51	154.08
126	56.69	188.98	15.91	198.31
127	28.44	56.75	0.1	1.55
129	2.85	252.88	4.71	249.51
130	49.88	114.28	1.68	29.64
131	50.46	50.27	0.08	4.38
132	9.62	6.39	0.06	6.3
133	155.67	197.02	3.89	114.4

The synthesis of methyl cinnamate is produced by the cinnamate/p-coumarate carboxylmethyltransferases (CCMTs; Kapteyn et al., 2007), in what appears to be a single gene scheme in *Ocium basilicum*. The data inspection showed a clear cut group of 87 plants that produced little or no methyl cinnamate (**Figure 2**) and a set of 23 that produced substantially higher levels. This data distribution fit perfectly to a 3:1 Mendelian model ($p = 0.3218$; **Table 2**) albeit with a dominant allele that did not produce methyl cinnamate and a recessive allele that produced this volatile. Thus, there is either a suppressor in *trans* or a dominant negative allele of the corresponding gene in the *A. majus* genetic background.

Finally, the synthesis of acetophenone is thought to be a degradation of ethylbenzene in bacteria. The degradation of 1-phenylethanol is caused by two enzymes a naphthalene dioxygenase and 1-phenylethanol dehydrogenase (Simon et al., 1993; Kniemeyer and Heider, 2001). We found what appeared to be a relative large number of lines producing very little acetophenone (41) indicating a possible effect of two genes. Indeed single gene models were significantly different from a 3:1 ($p = 0.002354$) or 1:2:1 ($p = 0.0001789$; **Table 2**). However, a 9:7 epistatic segregation was statistically possible ($p = 0.1966$) indicating the probable effect of two genes in the synthesis of acetophenone as proposed in bacteria. As found for β-ocimene, the *A. linkianum* alleles involved in acetophenone synthesis or its control, may not be null alleles resulting in small albeit detectable emissions.

Coding Region of *A. linkianum BAMT*

We decided to analyze the molecular structure of the *A. linkianumBAMT* allele segregating in the population. In order to identify possible lesions responsible for the loss of methyl benzoate emission we followed a candidate gene approach. Previous work has shown that the coding region of benzoic acid carboxymethyl transferase purified from petal tissue of *Antirrhinum* and expressed in *E. coli* can produce methyl benzoate, giving unequivocal biochemical support for the function of the corresponding gene product (Murfitt et al., 2000). Further, analysis of the protein expression has shown

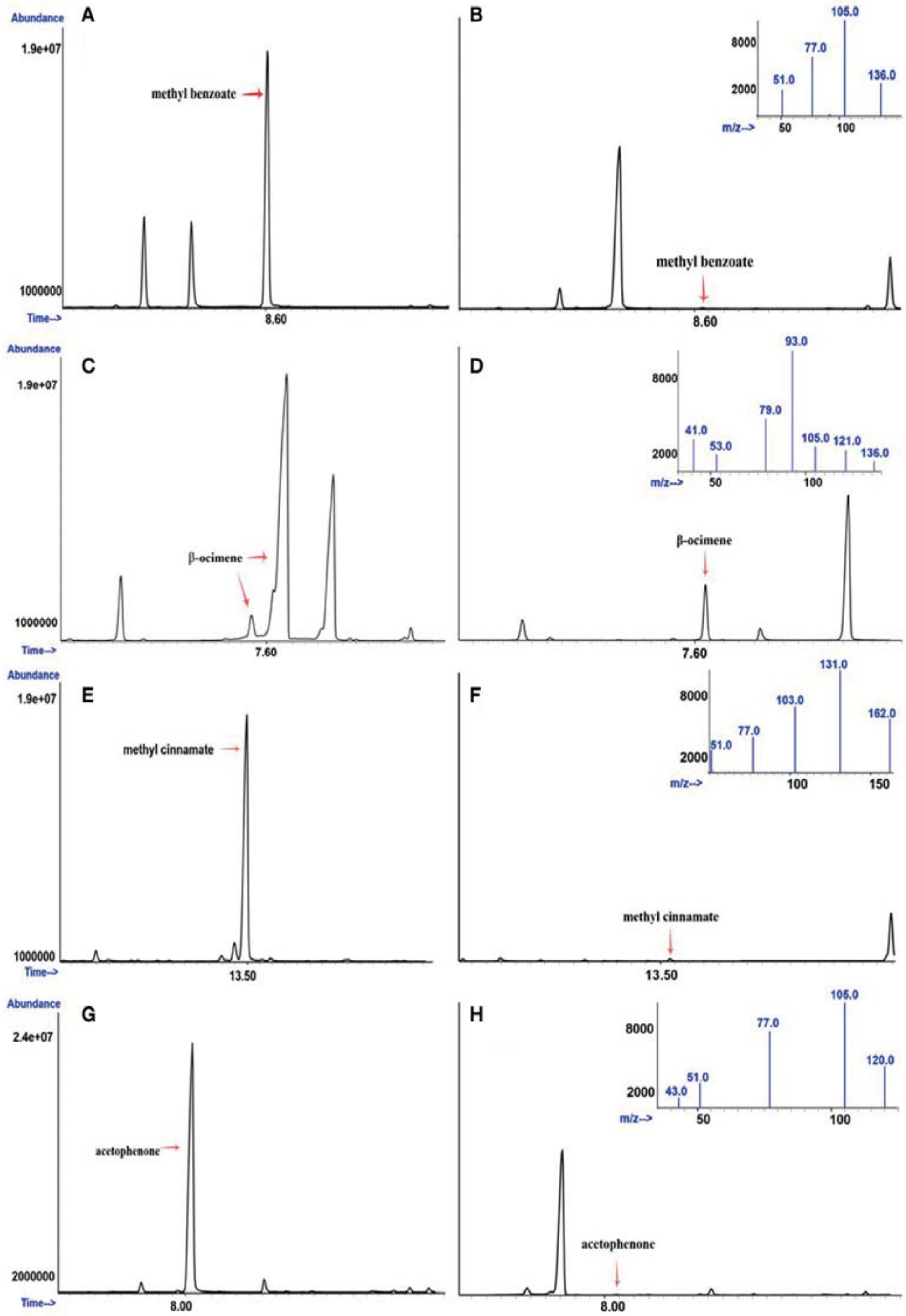

FIGURE 2 | Gas chromatogram and mass spectra data of plants differing in the emission of (A) high and **(B)** low methyl benzoate; **(C)** high and **(D)** low β-ocimene; **(E)** high and **(F)** low methyl cinnamate; and **(G)** high and **(H)** low acetophenone. The chromatograms reflect the different signal abundances as peaks. The *X*-axis corresponds to retention times in minutes.

that *A. majusBAMT* is expressed in petals (Kolosova et al., 2001b), the tissue that produces methyl benzoate. Despite the biochemical and cellular evidence, there was no genetic evidence that *A. majus BAMT* is the major gene involved in the synthesis of methyl benzoate. This is especially critical as there are two additional enzymes, salicylic acid carboxymethyl transferase (SAMT) and jasmonic acid carboxymethyl transferase (JAMT) with similar structure. Even though SAMT has a low Km for benzoic acid it is able to produce methyl benzoate *in vitro* (Negre et al., 2002; Effmert et al., 2005) and it may be able to produce some methyl benzoate *in vivo*.

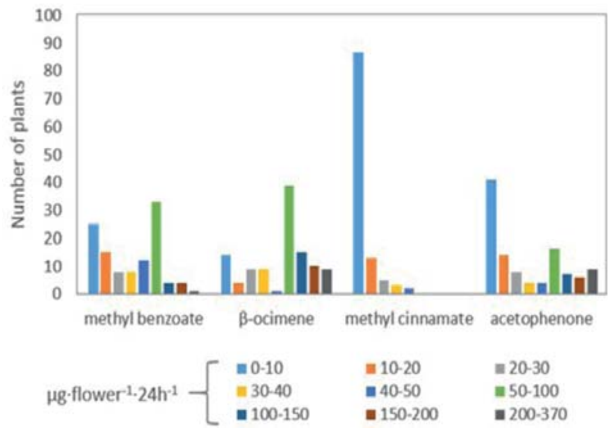

FIGURE 3 | Quantification of the number of plants emitting different quantities of methyl benzoate, β-ocimene, methyl cinnamate, and acetophenone.

TABLE 2 | Statistical analysis of the Mendelian segregation in an F2 population of methyl benzoate, β-ocimene, methyl cinnamate, and acetophenone in a cross of *A. majus* × *A. linkianum* (*n* = 110 plants).

Compound	Segregation model	Chi square *P*-value
Methyl benzoate	3:1 (85:25)	0.582
β-ocimene	3:1 (96:14)	0.002953
	3:1 (92:18)	0.03645
	3:1 (83: 27)	0.9123
	13:3 (96:14)	0.1056
Methyl cinnamate	3:1 (87:23)	0.3218
Acetophenone	3:1 (68:41)	0.002354
	1:2:1 (41:46:22)	0.0001789
	9:7 (68:41)	0.1966

We used the published sequence of *A. majus BAMT* to design primers to amplify the coding region of *A. linkianum BAMT* from gDNA in plants that were not producing methyl benzoate (Table S1). We used petal tissues from F3 plants producing large amounts of methyl benzoate and very low to undetectable levels (**Figure 4**). We could not detect *A. linkianum BAMT* expression in plants that did not emit methyl benzoate (**Figure 4**). The PCR primers developed to amplify a fragment of *A. linkianum* and *A. majus* turned out to be in a conserved region of the cDNA corresponding to the fourth exon, suggesting that the lack of amplification was not due to lack of annealing of the designed primers (Supplementary Figure 1).

The *BAMT* coding sequence in *A. majus* is composed of four exons (1110 bp). As the expression of *BAMT* in plants that do not produce methyl benzoate was undetectable, we used the genomic DNA of the parental *A. linkianum* to sequence the *A. linkianum BAMT* allele. We found a total of 40 SNPs in the coding region as compared to *A. majus*. These polymorphisms at the DNA level caused 15 amino acid changes at the protein level (**Figure 5**; Supplementary Figure 1). The mutations identified fell into two groups, one of conserved amino acid changes inside the BAMT/SAMT family of proteins and a second one of non-conserved amino acids. There were 10 amino acid changes in the first group but all the mutations corresponded to conservative amino acid substitutions. The amino acid His36 of *A. majus* BAMT is a proline in the proteins analyzed (**Figure 5**, **Table 3**) but was substituted by Arg in *A. linkianum* BAMT, that is conserved in the *Arabidopsis thaliana* JAMT (AT1G19640). The Ala69 from *A. majus* BAMT was conserved with *Clarkia breweri* SAMT but was substituted by a Thr in *A. linkianum* BAMT, that is conserved in most of the species analyzed. The second group of mutations comprised five amino acid changes in positions that are not conserved in the SHABAT proteins. The amino acid differences did not explain the complete lack of mRNA expression, indicating a different source of change.

We examined the exon-intron boundaries of the *AmajusBAMT* and *AlinkianumBAMT* genes. Both were identical at the exon-intron boundaries, except for a single base pair (596) within the third intron and four base pairs 3′ of the exon-intron boundary predicted by the *AmajusBAMT* annealing of genomic and cDNA sequences (Supplementary Figure 1). Using the *A. linkianum* sequence to predict possible exon-intron boundaries (Huang et al., 2006) for the corresponding interval where the 596 SNP occurs (bp400–700), gave as a result a splicing site identical to the exon-intron structure of *AmajusBAMT*. Thus, an alternative splicing that would produce a cDNA fragment lacking the last two exons, is not probable.

Genomic Structure of *A. linkianum BAMT*

We developed additional PCR primers based on the promoter region of *A. majus BAMT* and amplified 2125 bp of the promoter region of *A. majus*, and the corresponding region from the *A. linkianum* genome. The *A. linkianum* promoter was longer (2592 bp; **Figure 6A**) suggesting major changes in the promoter structure.

We divided the *A. linkianumBAMT* promoter in six fragments from 5′ to 3′ corresponding to six regions with distinct features (**Figure 6B**). We numbered the promoter with 1 as the adenine in the ATG of the *BAMT* CDS. The most distal fragment, −2592 to −2394 bp showed high homology to the *A. majus* promoter (−2125 to −1927 bp) and contained 10 SNPs (Supplementary Figure 2). The second fragment of *A. linkianum BAMT* (−2393 to −1763 bp) had a 631 bp insertion comparing to the *A. majus* promoter, located between the −1926 bp and the −1925 bp of *A. majus.* This insertion included genomic DNA that showed an extreme degree of homology (BLASTN e-114) with a fragment found in the promoter region of the *PLENA locus* (**Figure 7**; Bradley et al., 1993). This insertion contained an *IDLE* MITE transposable element (**Figure 6**; Cartolano et al., 2007; Schwarz-Sommer et al., 2010). The following fragment (−1762 to −1624 bp) of 138 bp length showed again high homology to the *A. majus* promoter (−1925 to −1786 bp) and had 6 SNPs. There was a 778 bp deletion in the *A. linkianum* promoter comprising the region between −1785 and −1006 bp from the *A. majus* promoter. The following fragment from −1623 to −1463 bp comprised 160 bp homologous to *A. majus* (−1007 to −852 bp) with 6 SNPs and a 4 bp insertion. The following fragment was an insertion of 608

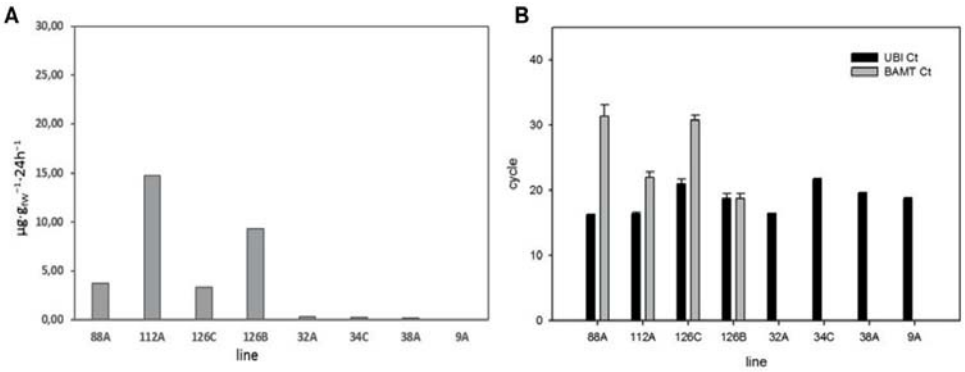

FIGURE 4 | (A) Emission of methyl benzoate in eight F3 lines with high and low emissions of methyl benzoate. **(B)** Quantitative PCR of BAMT gene expression of the high and low emitting lines. We used *AmUBC2* as positive control. Results are expressed as where the CT threshold was achieved.

bp comparing to the *A. majus* promoter, immediately after the −852 bp of *A. majus* promoter. This insertion had homology with the *VENOSA* genomic *locus* (Schwinn et al., 2006; BLASTN 2e-19). Furthermore the insertion was flanked by an 8 bp imperfect tandem duplication. Finally, the closest fragment to the start of transcription was −845 to −1 bp, homologous to *A. majus* −851 to −1 bp. It contained 45 SNPs, five deletions of 1–4 bp and three insertions of 1–4 bp.

Altogether the promoter of *A. linkianum BAMT* has two large insertions, one large deletion and several SNPs and indels, indicating that the complexity of changes may be responsible for the complete loss of expression in petals.

DISCUSSION

The identification of genes involved in the synthesis of scent and VOCs is an important effort that runs in parallel in a variety of plants. In crops, scent related traits include flavor and aroma and have undergone extensive research in many important plants such as rice (Lorieux et al., 1996; Singh et al., 2007; Golestan Hashemi et al., 2015), tomato (Klee, 2010; Klee and Tieman, 2013), strawberry (Zorrilla-Fontanesi et al., 2012), or trees such as peaches (Eduardo et al., 2013). The use of natural variation has helped first to define the genetic structure of the character and second to identify candidate genes involved in scent and volatile emission. Although scents are complex combinations of VOCs, the genetic structure of this trait has turned out to be composed of single genes and both regulatory genes such as the *MYB* gene *ODORANT1* from Petunia (Klahre et al., 2011) and a large number of enzymes have been identified using this approach. In this study we have analyzed the genetic structure of scent emission in two species of *Antirrhinum*, *A. majus* and *A. linkianum*, differing in the emission of four VOCs, methyl benzoate, methyl cinnamate, acetophenone, and β-ocimene (Weiss et al., 2016b). Our results show that the different VOCs displayed Mendelian segregations imputable to one or two genes.

The results of the segregation analysis coincided with the currently known biochemical models describing the catalytic reactions leading to the formation of the last step in the synthesis of methyl benzoate, β-ocimene, methyl cinnamate, and acetophenone. However, there are several aspects that may obscure the segregation analysis of scent VOCs. First, natural alleles may result in complete loss of function such as the *A. linkianum BAMT* identified in the current work. But small albeit detectable emissions of a compound such as methyl benzoate may be synthesized by a second enzyme such as *A. majus* SAMT, showing low affinity for benzoic acid (Effmert et al., 2005). The capacity to transform benzoic acid by SAMT seems to be enzyme specific as the tomato SAMT SlSAMT has a very low affinity for benzoic acid while the JAMT enzyme involved in the synthesis of methyl jasmonate can readily produce methyl benzoate (Tieman et al., 2010). Thus, the occasional emission of methyl benzoate could be the result of a family of proteins that have major affinities for their major substrate but maybe able to process additional metabolites with similar structures.

A second aspect is that incomplete loss of function i.e., leaky or weak alleles may result in segregations difficult to interpret as setting an emission threshold may not be straight forward. Indeed there are two possible gene models for β-ocimene synthesis, one based on a single gene, that would agree with the current biochemical model based on a single gene (Dudareva et al., 2003), while the second maybe based on two genes. As the *Antirrhinum* genome is not sequenced we cannot conclude if the first model is the correct one and further genetic and molecular analysis is required to resolve this issue.

The emission of methyl cinnamate appears to be recessive and the dominant allele may be a loss of function. The currently proposed biochemical model of methyl cinnamate synthesis is based on enzymes related to SHABAT carboxymethyl tranferases. This indicates that the loss of function maybe the result of a dominant negative translated gene product or the result of a local transposition event causing a double stranded RNA based gene silencing in trans.

The current model of acetophenone biosynthesis is based on the bacterial degradation of ethylbenzene by anaerobic catabolism where the last step is the degradation of 1-phenylethanol (Kniemeyer and Heider, 2001). This model may be conserved in higher plants where isotope labeling has shown that 1-phenylethanol is the major substrate for acetophenone

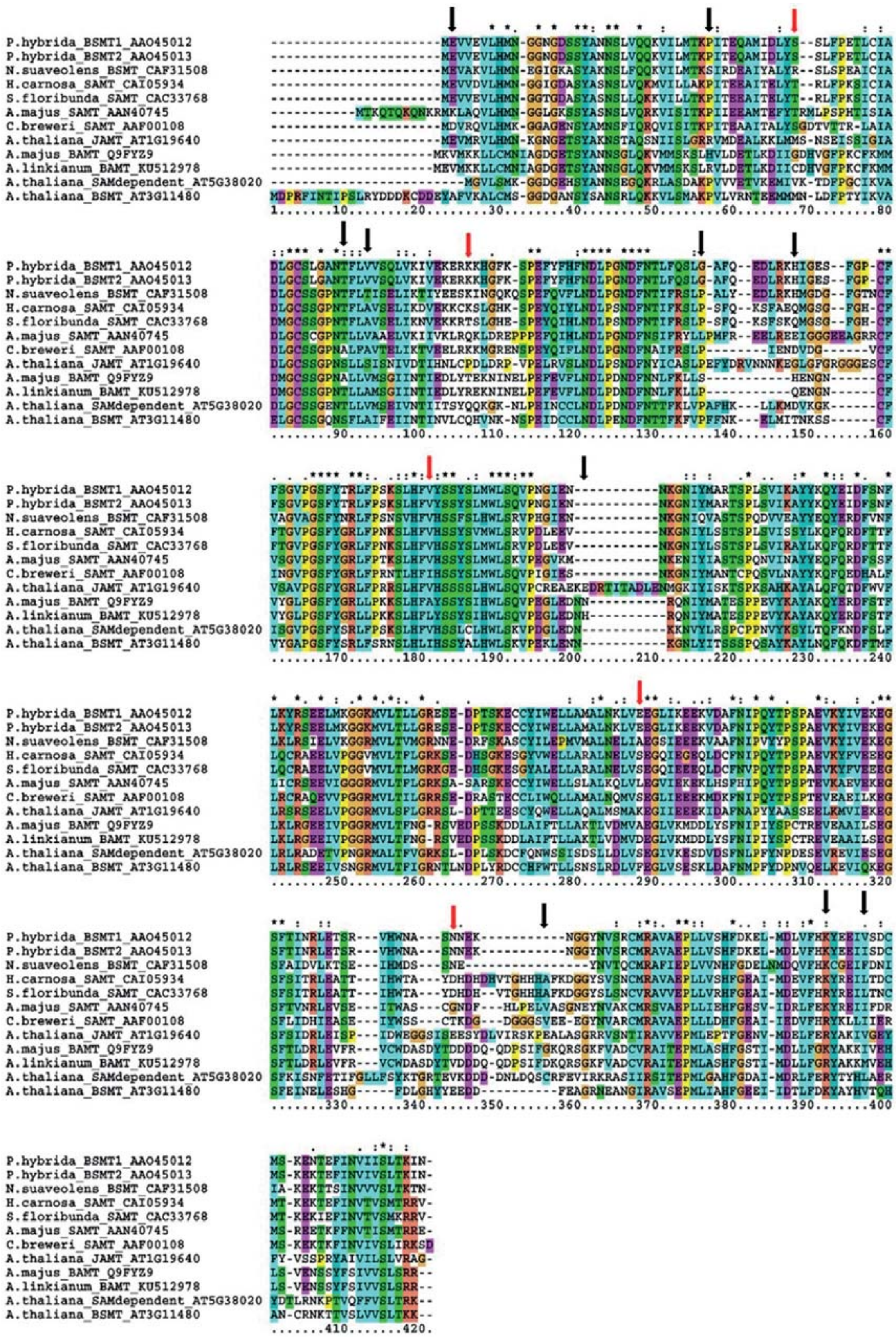

FIGURE 5 | Multiple sequence alignment of different SABATH family proteins. *Petunia hybrida*_BSMT1 (AAO45012), *P. hybrida*_ BSMT2 (AAO45013), *Nicotiana suaveolens*_BSMT (CAF31508), *Hoya carnosa*_SAMT (CAI05934), *Stephanotis floribunda*_SAMT (CAC33768), *Antirrhinum majus*_SAMT (AAN40745), *Clarkia breweri*_BAMT (AAF00108), *Arabidopsis thaliana*_JAMT (AT1G19640), *A. majus*_BAMT (Q9FYZ9), *A. linkianum*_BAMT (KU512978), *A. thaliana*_SAMdependent (AT5G38020), *A. thaliana*_BSMT (AT3G11480). Non-conserved amino acids with *A. linkianum* marked with red arrows, conserved amino acids with black arrows. Alignment performed with CLUSTALX (Larkin et al., 2007). Colors are default CLUSTALX color codings (Procter et al., 2010), corresponding to: blue hydrophobic; red positively charged, purple negatively charged, yellow small (P), cyan (Y and H), green polar, and orange (G). *Are conserved positions and: indicate amino acid conservative changes >0.5 in the Gonnet PAM 250 matrix, and a . indicates weak conservation <0.5 in the Gonnet PAM 250 matrix.

TABLE 3 | Aminoacid polymorphisms between *A. linkianum* and *A. majus* BAMT proteins and their position.

	Aminoacid														
A. majus	K	H	**G**	A	V	**T**	S	H	**A**	N	**A**	**D**	G	K	I
A. linkianum	E	R	**C**	T	A	**R**	P	Q	**L**	H	**D**	**V**	D	R	M
aa-Position	2	36	**47**	69	72	**85**	115	116	**143**	163	**239**	**292**	303	338	343

Methionine was in the position 1 in the coding sequence of both proteins. Polymorphisms in bold are non-conserved in BAMT/SAMT proteins.

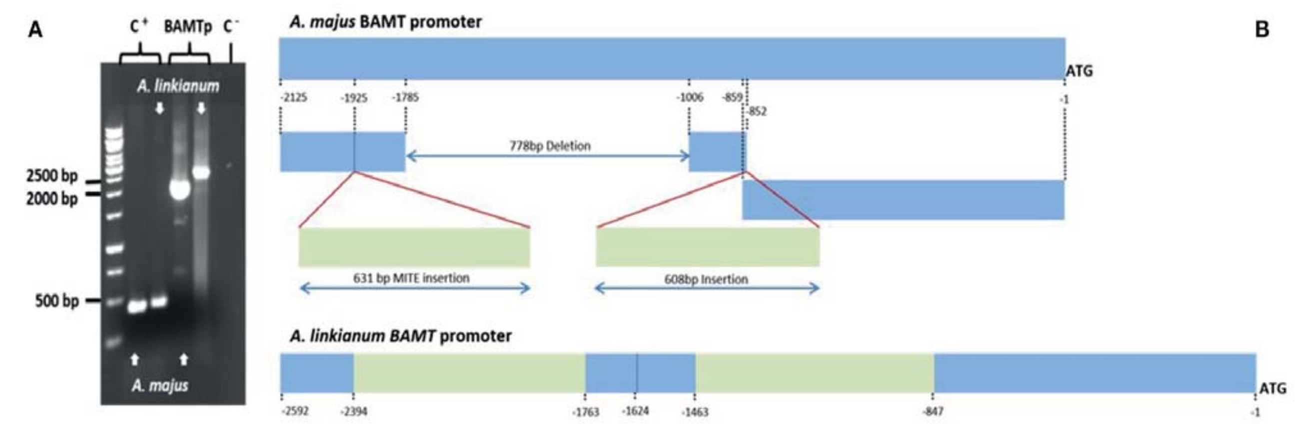

FIGURE 6 | Comparison of the molecular structure of the *A. majus and A. linkianum BAMT* promoter. (A) Electrophoresis of PCR product of *BAMT* promoter from *A. majus* and *A. linkianum. PLENA* as a positive control (C+) and water as a negative template (C−). **(B)** *A. majus* and *A. linkianum BAMT* promoters. Fragments in blue show high similarities between *A. majus* and *A. linkianum*. Green fragments show two major insertions in the *A. linkianum BAMT* promoter compared to the *A. majus BAMT* promoter.

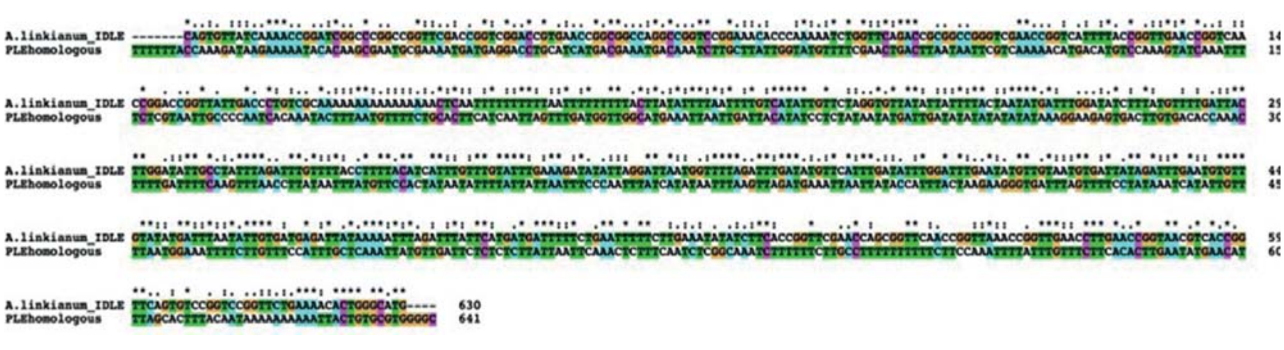

FIGURE 7 | Alignment of the two regions of the *A. majus PLE* promoter and the *A. linkianum BAMT* promoter showing high homology. DNA fragments were aligned with CLUSTALX. Coding corresponds to the default CLUSTALX. Color coding corresponds to T green, C magenta, A blue, G orange. *Correspond to conserved bases,: to T–A, C–A, or G–A changes and empty spaces to C–T, G–T, and G–C.

synthesis in *Camellia sinensis* (Dong et al., 2012). This suggests a conservation of the biochemical pathway in higher plants that probably perform this reaction under aerobic conditions. The segregation of acetophenone in the cross of *A. majus* × *A. linkianum* was most likely due to a two gene model, supporting the current evidence in bacteria where two enzymes are required to perform the synthesis of this volatile.

The lack of *BAMT* mRNA in plants that did not emit methyl benzoate led us to use the *A. majus BAMT* sequence to obtain information of the *A. linkianum BAMT locus.* The total of fifteen amino acid changes between *A. majus* and *A. linkianum BAMT* coding sequences is on one hand high, but is also restricted to either conservative changes or changes in non-conserved amino acids within a set of SABATH proteins. The intron-exon boundaries and predicted splicing sites of *A. majus* and *A. linkianum BAMT* were conserved. This indicates that the lack of mRNA of *A. linkianum BAMT* was not caused by alternative splicing differing between both alleles. The *A. majusBAMT* gene expression is circadian regulated (Kolosova et al., 2001a). We had sampled the flowers for mRNA at the highest level of recorded expression, i.e., roughly at T6 of subjective time. As we neither found *A. linkianum BAMT* expression at this point, nor methyl benzoate emission in a 24 h interval, our assumption is that *A.*

linkianumBAMT does not produce mRNA at other times of the day.

However, as the mRNA was undetectable in *A. linkianum* or the corresponding siblings we pursued further to analyze the regulatory region of *A. linkianum BAMT*. The mutations found in the promoter of the *A. linkianum BAMT* gene comprise three major changes including what appears to be an event of non-homologous recombination causing an insertion of a 630 bp fragment that is with high probability originated at the *PLENA locus*. The insertion contains an *IDLE* transposable element that is also present in the *PLENA locus* (Cartolano et al., 2007). The second modification is caused by a 608 bp insertion with low homology to the *VENOSA locus* (Schwinn et al., 2006), flanked by two 8 bp imperfect repeats. These type of mutations maybe caused in an original promoter by a patch-mediated double-strand break induction and repair mechanism (Vaughn and Bennetzen, 2014). We cannot determine if the 778 bp deletion occurred linked to the aforementioned events or happened independently. However, small changes such as 4 bp deletions can give raise to weak hypomorphic alleles such as *deficiens chlorantha* (Schwarz-Sommer et al., 1992). So our assumption is that the complex rearrangement of the *A. linkianum* regulatory region creates what appears to be a null allele in terms of mRNA expression. The low emission of methyl benzoate by some of the F2 recessive lines can be explained by the non-specific production of this compound by SAMT (Effmert et al., 2005). Methyl salicylate synthesis is activated in response to stress. The gene encoding for SAMT is induced by salicylic acid and jasmonic acid in *Antirrhinum* petals (Negre et al., 2002). Although the reported Km of SAMT is over 100-fold lower for benzoic acid than salicylic acid it may be responsible for the small amounts of methyl benzoate emission that we could detect in some samples. We have not tested the hypothesis of a direct involvement of *A. majus BAMT* on the synthesis of methyl salicylate or methyl jasmonate, as we have not performed experiments under stress conditions aimed to activate these pathways. Nevertheless, methyl salicylate is amongst the most common floral scent VOCs (Knudsen et al., 2006), Our data shows that complex scent profiles can be resolved to combinations of Mendelian genes involved in synthesis or control of scent components. The high transposon activity of the *Antirrhinum* genus may be involved in the diversity of profiles and may play a role in local adaptation to pollinators.

AUTHOR CONTRIBUTIONS

VR-H, BH, JW, and ME-C designed experiments; performed experiments; analyzed the data; corrected and approved the final manuscript. VR-H, JW, and ME-C wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00027/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Genetic analysis of natural variation in Antirrhinum scent profiles identifies BENZOIC ACID CARBOXYMETHYL TRANSFERASE as the locus controlling methyl benzoate synthesis

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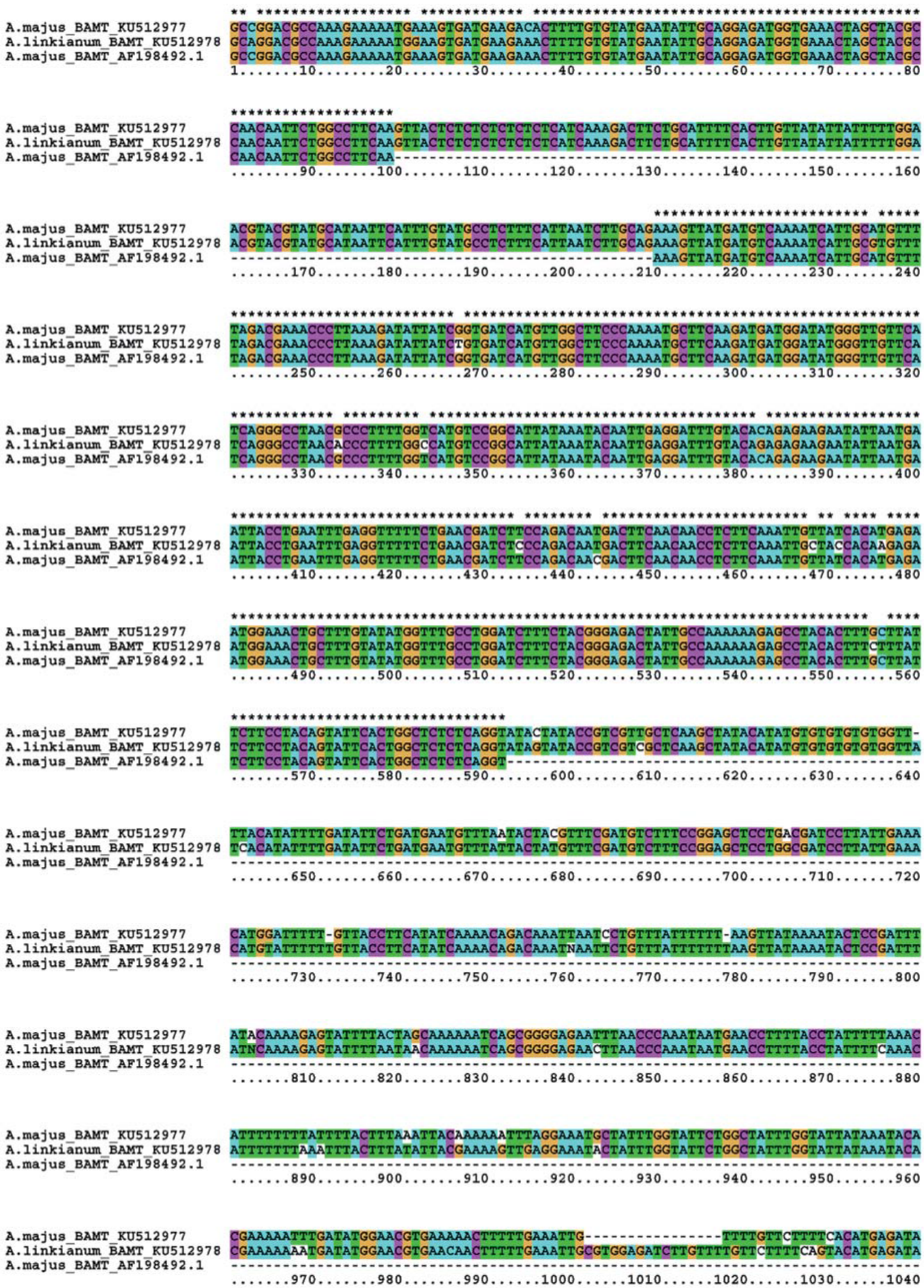
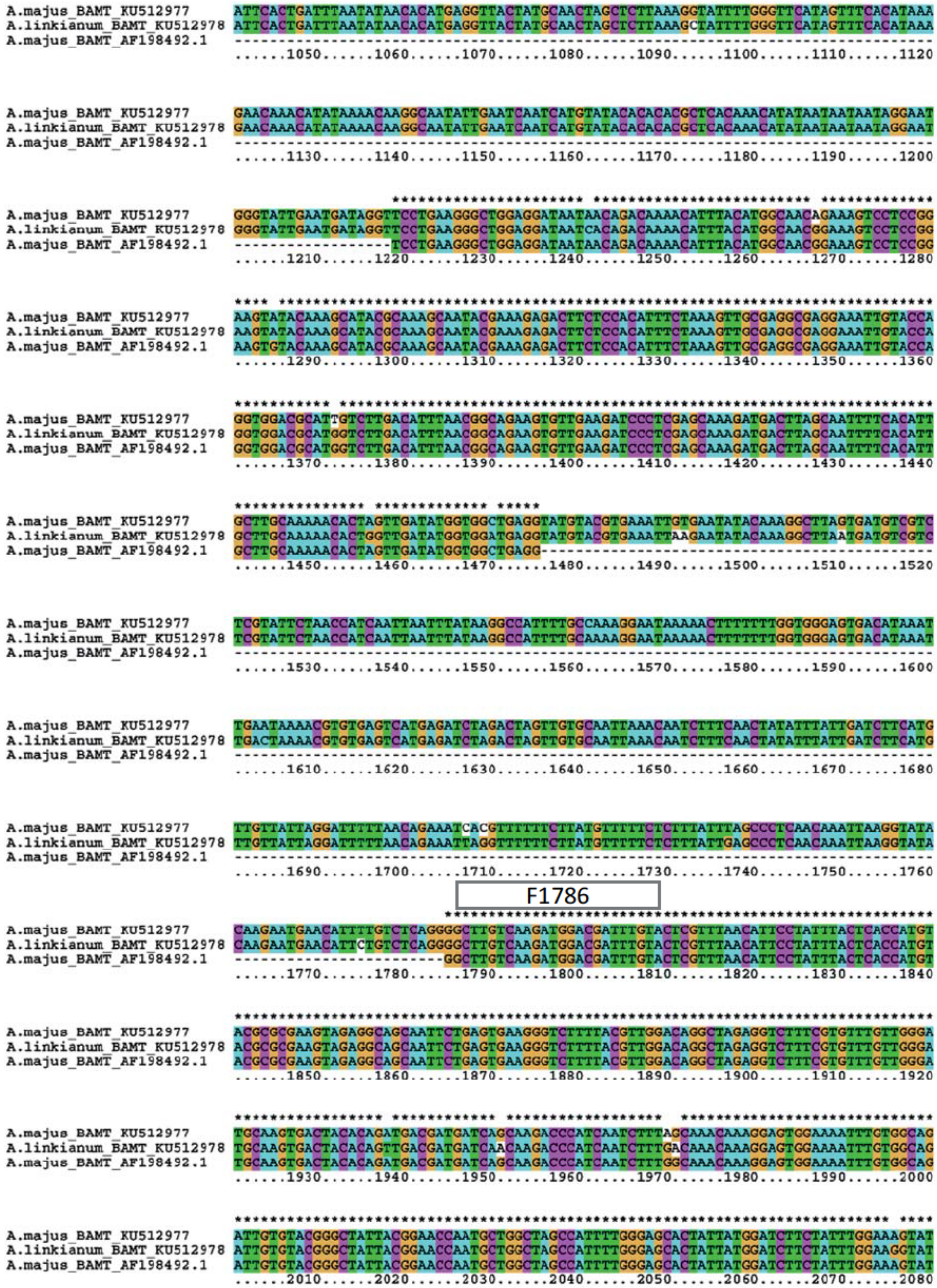
1 Supplementary Tables

Table S1. List of primers used. BAMTprom Forward and Reverse were used for amplifying the BAMT promoter in *A. majus* and *A. linkianum* from gDNA. BAMT_F1786 and BAMT_R2408 were used for amplifying a transcribed region of BAMT. PLENA and UBIQUITIN primers were used as positive controls.

Primer Name	Sequence
BAMTprom Forward	AACCGATGAATTTACGCACA
BAMTprom Reverse	TTCTACTCGGGTATAACTTCTAACG
PLENA Forward	AAATGCTTTCCCAGTTGCTCAACTGC
PLENA Reverse	TATCAGGAGCTTGAGCTGCACAATGC
BAMT_F1786	GCTTGTCAAGATGGACGATTTGTAC
BAMT_R2408	TTCCATTACTTCTGCTATAAAAATAGTACT
UBIQUITIN Forward	TGGAGGATGGAAGGACTTTGG
UBIQUITIN Reverse	CAGGACGACAACAAGCAACAG

2 Supplementary Figures

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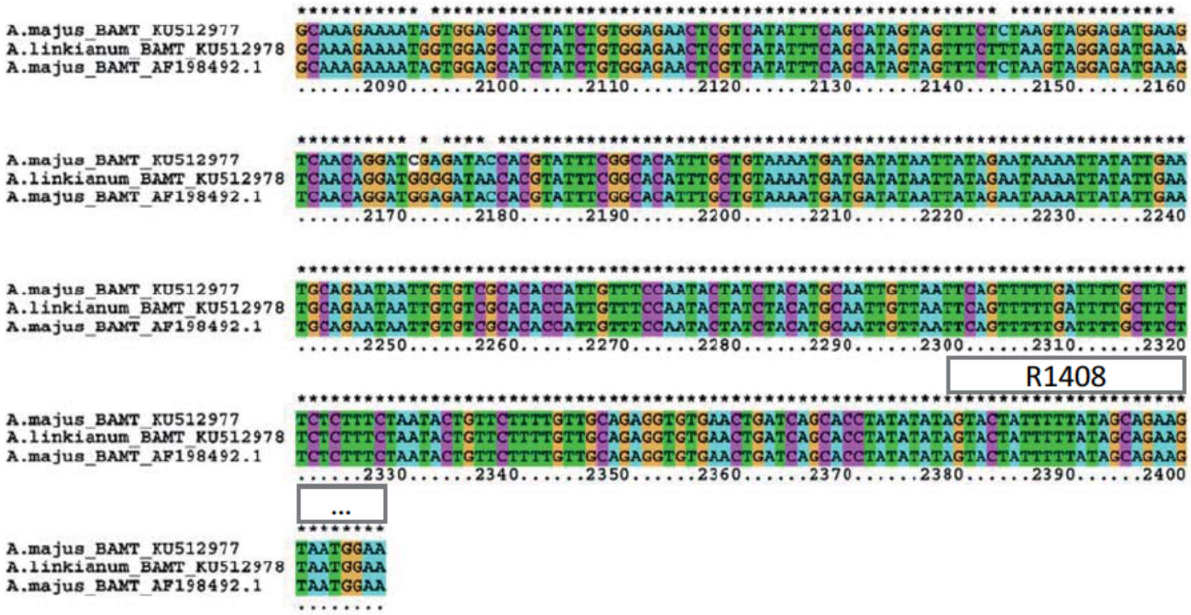
A.linkianum_BAMT_KU512978
A.majus_BAMT_AF198492.1
F1786



Supplementary Figure 1. Clustal-X alignment between *A. majus* genomic sequence (KU512977), *A. majus* coding sequence (AF198492.1) and *A. linkianum* genomic sequence (KU512978) of *BAMT*.

```
>A.majus_BAMTp          AACCGATGAATTTACGCACATTTAATAATTCTAAAAACAATTTAAATTTT
>A.linkianum_BAMTp      AACCGATGAATTTACGCACATTTAATAGTTATAAAAAAAATTTAAAATTT
                        *************************** ** ****** ******** ***

>A.majus_BAMTp          TATTTGGTTAACCATCAATCATCACATGCATTATGATCCTATCATAAATT
>A.linkianum_BAMTp      TATTTCTTTAACCATCAATCATCACACCCATTATGATCCTACCATAAATT
                        *****  *******************  ************* ********

>A.majus_BAMTp          ATTATATATAAATCTAACAATACTATAGAAAGTGACATGTGATTTGGCAC
>A.linkianum_BAMTp      ATTATATATAAATCTAACAATACTATAGAAAGTGACATGTGATTTGGCAC
                        **************************************************

>A.majus_BAMTp          ATAACTAATTATTGACCTTCTGAATGGTCTTGTCCACCCGTGGAGGCATG
>A.linkianum_BAMTp      ATAACTAATTATTGACCTTCTGAATGGTCTTGTCCACCGGTGGAGGCAT-
                        ************************************** ********** 

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      CAGTGTTATCAAAACCGGATCGGCCCGGCCGGTTCGACCGGTCGGACCGT

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      GAACCGGCGGCCAGGCCGGTCCGGAAACACCCAAAAATCTGGTTCAGACC

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      GCGGCCGGGTCGAACCGGTCATTTTACCGGTTGAACCGGTCAACCGGACC

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      GGTTATTGACCCTGTCGCAAAAAAAAAAAAAAAAACTCAATTTTTTTTTA

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      ATTTTTTTTTACTTATATTTTAATTTTGTCATATTGTTCTAGGTGTTATA

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      TTATTTTACTAATATGATTTGGATATCTTTATGTTTTGATTACTTGGATA

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      TTGCCTATTTAGATTTGTTTTACCTTTTACATCATTTGTTTGTATTTGAA

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      AGATATATTAGGATTAATGGTTTTAGATTTGATATGTTCATTTGATATTT

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      GGATTTGAATATGTTGTAATGTGATTATAGATTTGAATGTGTTGTATATG

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      ATTTAATATTGTGATGAGATTATAAAAATTTAGATTTATTCATGATGATT

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      TTTCTGAATTTTTCTTGAAATATATCTTCACCGGTTCGAACCAGCGGTTC

>A.majus_BAMTp          --------------------------------------------------
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>A.majus_BAMTp        ------------------------------CATTGCTTAGAAATTTAGAG
>A.linkianum_BAMTp    TCCGGTCCGGTTCTGAAAACACTGGGCATGCATTGCATAGAAATTTAGAG
                                                    ****** *************

>A.majus_BAMTp        CCCCTCAAATTCCTAAAATGCCTTATTAAAGGGGAGGTGGGTTATTGATA
>A.linkianum_BAMTp    CCCCTCAAATTCCTAAAATGCCTTATTAAATAGAAGGTGGGTTATTGATA
                      ******************************  * ****************

>A.majus_BAMTp        TATGTCATGTACAGACCGGAAAGATACCATATAGAAATTTTGAGTCTCCT
>A.linkianum_BAMTp    TATGTCATGTACAGACCGGAATGATACCATATAAAAATTTTGAGTCTCCT
                      ********************* *********** ****************

>A.majus_BAMTp        CAACTCCTAAAAGACCTTGTTGTTCCCTTGGTTGTTCCCTTGTTCAAAAA
>A.linkianum_BAMTp    CAACTCCTAAAAGACCTTAT------------------------------
                      ****************** *

>A.majus_BAMTp        CCACAATAATAAACTAGAGAAATAAAATGATTTTACAACACTTTAACATA
>A.linkianum_BAMTp    --------------------------------------------------

>A.majus_BAMTp        AAATAGATATTACATCAACTCATGAATTGGATGAATGTTACCTCGAAGTC
>A.linkianum_BAMTp    --------------------------------------------------

>A.majus_BAMTp        GATAATTAGTTAATTACCAAAAATGACACAATATAATAGTCAGGATGAGG
>A.linkianum_BAMTp    --------------------------------------------------

>A.majus_BAMTp        GGTGGACTAGGTCTCTGCTCTCTTAAAGACGGTTTTGCGCCCCTACAGAT
>A.linkianum_BAMTp    --------------------------------------------------

>A.majus_BAMTp        TTTTATCCGATGCAGTAGGCTACTGACACGTCCGTCTCCGAGATACAATA
>A.linkianum_BAMTp    --------------------------------------------------

>A.majus_BAMTp        GCCCAACTTTTCTTGTTGTAAACTTCGCTCTAAGTCTGCACTACTTAAGG
>A.linkianum_BAMTp    --------------------------------------------------

>A.majus_BAMTp        AAGTCGAAGCGTAGCCCAACACTTTTCTTTGTCCATAAATCTTTTCTCAT
>A.linkianum_BAMTp    --------------------------------------------------

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>A.linkianum_BAMTp    --------------------------------------------------

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>A.linkianum_BAMTp    --------------------------------------------------

>A.majus_BAMTp        CTTCAAAATGATATGCAAGACCACTATTTATAGTGTAAAACTCACTAGTC
>A.linkianum_BAMTp    --------------------------------------------------

>A.majus_BAMTp        TTGTAACTCCAAAATAATTAGAGAATTAATATATAATATTATTACACTAT
>A.linkianum_BAMTp    --------------------------------------------------
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>A.majus_BAMTp          ATAAATATGATAGTAATGGGGGTGTTTAATTCACACAATAAAAGAGGTG
>A.linkianum_BAMTp      --------------------------------------------------

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>A.linkianum_BAMTp      --------------------------------------------------

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>A.linkianum_BAMTp      --------------------------------------------------

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>A.linkianum_BAMTp      ------------------------------------------------TA
                                                                        **

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>A.linkianum_BAMTp      AGAAGAAAATAGAATGTTAATTAATATACCAAACCCAAATTCCTTCTCAA
                        ** ************ *** ************** ******* *******

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>A.linkianum_BAMTp      GCATTAATTAATATTGCTAGATTTCATCAAACAACATTAATTTAGTTGT
                        ********    ***************** ********************

>A.majus_BAMTp          CCCCTATTTTTCCATTTTCTAAAGGTAGAGTGCTTTCACGTGCGACATGA
>A.linkianum_BAMTp      CCCCTATTTTTCCATTTTCTAAAGGTAGAGTGCTTTCACGTGCGACATGA
                        **************************************************

>A.majus_BAMTp          TTTACACC------------------------------------------
>A.linkianum_BAMTp      TTTTCACCATTAAAAGAATAAAATTGTAAGTAGATTTGTGATGAATTACA
                        *** ****

>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      TCTACTTAACTATATTGAATATTTTGTGATATGCGATACTTTATAAGTAC

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>A.majus_BAMTp          --------------------------------------------------
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>A.majus_BAMTp          --------------------------------------------------
>A.linkianum_BAMTp      AAACTCACATAGTGTAGGAGAGTCTTTATGTAAATCATCATCTGCAATTA

>A.majus_BAMTp          --------------------------------------------------
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>A.majus_BAMTp          --------------------------------------------------
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>A.majus_BAMTp          --------------------------------------------------
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>A.majus_BAMTp          --------------------------------------------------
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>A.majus_BAMTp          --------------------------------------------------
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>A.majus_BAMTp          --------------------------------------------------
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>A.majus_BAMTp          ------------------------TAACTCGAATTGAGCAATCATACTCG
>A.linkianum_BAMTp      TCTGAATACGCCCCTGTTTGCATCTAACTCGACTTGAGCAATCATACTCG
                                        *** ** ********* *****************

>A.majus_BAMTp          TGATAGATAAGTTAATTTCACATGTATTACTCTTTTTAAATTGACCAAAA
>A.linkianum_BAMTp      TGATAGACAAGTTAATTTCACATGTATCACTGTTTTCAAATTGACCCAAA
                        ******* ******************* *** **** ********* ***

>A.majus_BAMTp          AAAAATGTCATACTATAAGATTAAACTGGGAAGTGGGGGGCGTATCTACT
>A.linkianum_BAMTp      AAAAAAGTCATACTATAAGATTAAA-TGGGAAGTGGGGGGCGTATCTACT
                        ***** ******************* ************************

>A.majus_BAMTp          TAGGGTACTCCAGCCCTCTTATATATATAATGTATTATATATATATATAT
>A.linkianum_BAMTp      TAGGGTCCAGCTAGCCTCTTATATATATA---TAT-ATATATATATATAT
                        ****** *  *   ***************   *** **************

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>A.linkianum_BAMTp      ATATTCAAAAAGGAAAAAAAATATTTATTGAAATTGTTAAAGGTTACAAA
                        ***  ******** ************************************

>A.majus_BAMTp          TTCAATTTTCAATAAAGCTTATCGACAAAATCCCAAACTATAGAAAATTA
>A.linkianum_BAMTp      TTCAATTTTGAATAAAGCTTATCGACAAAATCCCAAACTATAGAAAATTA
                        ********* ****************************************

>A.majus_BAMTp          AGTTCTCTTCAACGCCCAACAATCAAACGTATTGACAAAATCTTTATGTT
>A.linkianum_BAMTp      AGTTTTCTTCAACGCCCAACAATCAAATGTATTGACAAAATCTTTATGTT
                        **** ********************** **********************

>A.majus_BAMTp          CTTGGTAAAAATTTGTAGTAGTCTGAAATTTTAATAATGTGACAAAGGAA
>A.linkianum_BAMTp      ATTG-TAAAATTTTGGAGTAGTCTGAAATTTTAATAATGTGACAAAGGAA
                         *** ***** **** **********************************

>A.majus_BAMTp          CATAAGTAAGCAAATAGTGAA----AAACTAAATATATATTAACTCATAT
>A.linkianum_BAMTp      CATAAGTAAGCAAATAGTGAATAGAAAACTTAATATATATTAACTCATAT
                        *********************    ***** *******************

>A.majus_BAMTp          TTTCAATTATTATATTCATGTGTTGTTAAGTGATTAATATATCATTTACC
>A.linkianum_BAMTp      TTTCAAATATTATATTCATGTGTTGTTAAGTGATTAATATATCATTGACC
                        ****** ******************************************* ***

>A.majus_BAMTp          ATTAGTATTTTTGACTTTTTCAATTTTTTGTTATTTAATTTGTTTTTGT
>A.linkianum_BAMTp      ATTAGTATTTTTAACCTTCAATTTTTTTTGTTATTTAATTTGTTTTTGT
                        ************ ** **     ***************************
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>A.majus_BAMTp          GTTATATATCACTTTTATATTATTAT-TATTACTTGTTTTACCAAAAAAA
>A.linkianum_BAMTp      GTTATATATCACTTTTATATTATTAT TATAACTTGTTTTACCAAAAAAA
                        ************************** *** *******************

>A.majus_BAMTp          TATTTA AATTTCTTATATTGTGTTTCTTATATTTTAATTTCTTAAATTA
>A.linkianum_BAMTp      AATTTT AAATTCTTATATTGTGTTTCTTATATTTTAATTTCTTAAATTA
                         ****  ** ****************************************

>A.majus_BAMTp          CATACCTTTTTCATAGTACTTAAAAAATATATCCGTTGGATTAGTCCAAC
>A.linkianum_BAMTp      CATACTTTTTTCATAGTACTTAAAAAATATATCCGTTGGATTAGCCCAAC
                        ***** ************************************** *****

>A.majus_BAMTp          GCAGACTGAATTCCTGGATACGCCCTCGTGTACATACGTGCTATATTAGA
>A.linkianum_BAMTp      GCAGACTGAATTCCTGGATACGCCCTCGTGTACATACGTGCCATATTAGA
                        ***************************************** ********

>A.majus_BAMTp          AAATAGGAAGAGCAAACTATCAAGTATTTGTAAGTTGTAGCTGCCGAACA
>A.linkianum_BAMTp      AAATAGGAAGAGGAAACTATCAAGTATTTGTAACTTGTAGCTGCCGAACA
                        ************ ******************** ****************

>A.majus_BAMTp          ATTTGGTTACTGAATTATATATA ATATGTGAATCTATTTGCTGGTAAGC
>A.linkianum_BAMTp      ATTTGGTTATTGAATTATATATA    TGTGAATCTATTTGTAGGTAAGC
                        ********* *************    **************  *******

>A.majus_BAMTp          GTTAGAAGTTATACCCGAGTAGAAGAATTAA
>A.linkianum_BAMTp      GGTAGAAGTTATAACCGAGTAGAAGAATTAA
                        * *********** *****************
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Supplementary Figure 2. *A. majus* vs *A. linkianum BAMT* promoter. Key color: green-insertions, blue-deletions, yellow-repetitions.

Chapter III

A Comparison of Semi-Quantitative Methods Suitable for Establishing Volatile Profiles

Ruiz-Hernández *et al. Plant Methods (2018) 14:67*
https://doi.org/10.1186/s13007-018-0335-2

Plant Methods

METHODOLOGY

Open Access

A comparison of semi-quantitative methods suitable for establishing volatile profiles

Victoria Ruiz-Hernández[1,2], María José Roca[3], Marcos Egea-Cortines[1,2] and Julia Weiss[1,2*]

Abstract

Background: Full scent profiles emitted by living tissues can be screened by using total ion chromatograms generated in full scan mode and gas chromatography–mass spectrometry technique using Headspace Sorptive Extraction. This allows the identification of specific compounds and their absolute quantification or relative abundance. Quantifications ideally should be based on calibration curves using standards for each compound. However, the unpredictable composition of Volatile Organic Compounds (VOCs) and lack of standards make this approach difficult. Researchers studying scent profiles therefore concentrate on identifying specific scent footprints i.e. relative abundance rather than absolute quantities. We compared several semi-quantitative methods: external calibration curves generated in the sampling system and by liquid addition of standards to stir bars, total integrated peak area per fresh weight (FW), normalized peak area per FW, semi-quantification based on internal standard abundance, semi-quantification based on the nearest *n*-alkane and percentage of emission. Furthermore, we explored the usage of nearest components and single calibrators for semi-quantifications.

Results: Any of the semi-quantification methods based on a standard produced similar or even identical results compared to quantification by a true-standard for a compound, except for the method based on standard addition. Each method beholds advantages and disadvantages regarding level of accuracy, experimental variability, acceptance and retrieved quantities.

Conclusions: Our data shows that, except for the method of standard addition to the biological sample, the rest of the semi-quantification methods studied give highly similar statistical results. Any of the methodologies presented here can therefore be considered as valid for scent profiling. Regarding relative proportions of VOCs, the generation of calibration curves for each compound analysed is not necessary.

Keywords: GC–MS, HSSE, Internal standard, Calibration curve, Quantification, Scent profile, Stir bars, Twisters, VOCs

Background

The emission of Volatile Organic Compounds (VOCs) is a biological feature of bacteria, fungi, plants and animals. They play a key role in interaction between individuals of the same and other species, genera and kingdoms [1–3]. The number of identified VOCs emitted in nature is constantly increasing as the analytical techniques improve and biodiversity is scrutinized for its chemical diversity. Numerous ecological studies are focusing on VOC functions i.e. the mediation of plant defence by volatile compounds in plant communities [4].

VOCs emission by plants can be very variable, especially in flowers where different compounds comprising a specific scent profile may be counted in dozens [5]. Bioactivity of VOCs emitted by flowers is diverse and not fully understood yet. While some of these compounds are known to have an effect over pollinator attraction, others may act as repellents [6, 7].

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In order to identify as many compounds as possible in a given sample, researchers use HSSE, and GC–MS using TICs when screening scent profiles. A scent profile is understood as the combination and proportion of VOCs which is conserved for a certain set of samples and reflect a combination of the genotype and environmental conditions.

The composition of floral scent profiles is robust for a given species [8]. Most scientists distinguish major and minor VOCs, where major compounds are emitted in higher quantities and can be interpreted as the characteristic footprint of a species. Minor compounds contribute to the fine tuning of the ultimate bouquet. The VOCs emitted by a plant organ changes depending on the time of the day, physiological stage and biotic and abiotic external factors [9–11].

An appropriate quantification of compounds ideally requires the use of a specific standard for each compound present in the sample [12, 13]. Considering that plant VOCs may be present in dozens, their quantifications based on the inclusion of standards for each VOC increases the economic costs considerably [14]. An additional problem is that pure standards may not be available for most compounds [15, 16]. Furthermore, while some scent profiles are composed of VOCs emitted constitutively, other compounds are emitted only under certain circumstances, and the appropriate standards are therefore not known a priori [17]. Finally, there are VOCs that are known for having several isomers with differing Chemical Abstract Service number and therefore a potential standard. As a result, performing appropriate quantifications of VOCs emitted by plants is not straightforward.

Due to the high costs and lack of standards for every chemical compound, other methods that allow semi-quantification of compounds can be used. The most common method is the usage of single internal standards for extrapolating quantities [10, 18–20]. Moreover, several internal standards can be used for semi-quantifying nearest or similar components within the chromatogram [15, 21]. However, the use of internal standards has some drawbacks related to the response of VOCs during the chromatographic analysis, such as differential binding to the adsorption fiber [22, 23].

Here we compared external calibrators obtained by adding standards to the sampling system, external calibrators by liquid addition to stir bars, calculation of integrated peak area·gFW^{-1}, calculation of normalized peak area·gFW^{-1}, semi-quantification based on internal standard, semi-quantification based on external calibrator area using the NearestRT *n*-alkane. Our results indicate that except for the semi-quantification by standard addition to the biological sample, the rest of the methods studied give highly similar statistical results. Furthermore, results indicate that the use of a standard for each VOC analysed in the context of scent profiles studies can be omitted.

Methods

Plant material and VOCs collection

We used completely developed 3–4 days old flowers of the *Antirrhinum majus* inbred line 165E [8, 24] in order to generate the raw data which were then used to compare semi-quantification methods. Additionally, a flower scent profile was generated for *Petunia x hybrida* line Mitchell. The sampling system consisted in flowers placed inside a beaker with 4 ml of 5% sucrose in distilled water, supported by a glass slide, and a stir bar was attached to the border of the beaker with a stainless-steel paperclip. The beaker was then placed in a 2-l desiccator (Fig. 1).

Flowers for CG-MS analysis were kept under conditions of 12 h light and 12 h dark at 23 °C and 18 °C, respectively, in a growth chamber (Sanyo MRL 350). In case of *A. majus*, stir bars sampled the floral volatiles of 3 flowers in 3 different desiccators during 12 h of light or 12 h of dark periods. In case of *Petunia x hybrida*, stir bars sampled floral volatiles for 4 or 24 h, sampling times applied in circadian rhythm studies [25]. The VOC profile of *A. majus*, is based on compounds which appeared unanimously in the day and night replicas (Table 1). Contaminants were identified and omitted in subsequent analyses.

We used 10 mm long Twisters™ (Gerstel, Mülheim an der Ruhr, Germany) (stir bars), covered with a 0.5 mm film of polydimethylsiloxane (PDMS). We also tested dual-phase stir bars (ethylene glycol and silicone) (Gerstel, Mülheim an der Ruhr, Germany). Both types of stir

Fig. 1 Sampling system for VOCs in HSSE

Table 1 Chromatographic parameters for *A. majus* VOCs analysed in column HP5 MSVi

Retention time	Compound	CAS	LRI	LRI bibliography	Reference
2.23	Methyl 2-methylbutanoate	868-57-5	805	774	[26]
5.58	β-myrcene	123-35-3	996	991	[27]
6.62	Ocimene	6874-10-8	1044	1038	[28]
7.19	Acetophenone	98-86-2	1071	1065	[27]
7.77	Methyl benzoate	93-58-3	1099	1091	[27]
7.96	Nonanal	124-19-6	1107	1104	[29]
9.03	Acetophenone, 2′-hydroxy	118-93-4	1167	1160	[26]

bars were conditioned for adsorption according to manufacturer indications.

Compounds adsorbed by the stir bars were analysed by GC–MS in a gas chromatograph HP-6890N coupled to a 5975 mass spectrometer (Agilent Technologies, Palo Alto, USA) combined with a TDU and cooling injector system (CIS4) (Gerstel, Mülheim an der Ruhr, Germany).

Desorption was carried out by heating from an initial temperature of 40° to 250 °C at 100 °C min^{-1} with 5 min hold time on splitless mode. Desorbed compounds were captured in a cool trap at −100 °C. This process was automated by using a multipurpose sampler MPS2XL (Gerstel, Mülheim an der Ruhr, Germany).

Chromatographic separation was done in a HP5MS-UI column (Agilent Technologies, Palo Alto, USA) with helium as gas carrier in constant pressure mode and split ratio 1:50. Initial temperature was 50 °C, increasing at a ratio of 5 °C min^{-1} until 70 °C held 1 min. In the next step, temperature was increased until 240 °C at 10 °C min^{-1} held for 15 min.

The mass spectrometer operated at 70 eV ionization voltage. Source and quadrupole temperatures were 230 and 150 °C, respectively. Mass range was 30.0 to 450.0 uma at 4 scan/s. MSD transfer line was maintained at 280 °C.

We used ChemStation software (version E.02.02 SP1, Agilent Technologies, Palo Alto, USA) to acquire chromatograms. Compounds were qualitatively identified by comparison with mass spectral database Willey10th-NIST11b (Agilent Technologies, Wilmington, USA), considering match qualities above 90%. We used ocimene, acetophenone, methyl benzoate and methyl cinnamate (Sigma-Aldrich, W353901, 42163, 18344 and 96410, respectively) as standards. Methanol was used as solvent for dilution of standards (Panreac, 361091). Linear retention indexes (LRI) were calculated as a parameter for identifying compounds by comparing with retention times (RT) of C8-C20 alkanes (Sigma Aldrich, 04070), analysed under the same chromatographic conditions (Table 1) [30].

Semi-quantitative methods of VOCs analysis

We analysed raw data using: (1) external calibrators obtained by adding standards to the sampling system, (2) external calibrators obtained by liquid addition to stir bars, (3) calculation of integrated peak area·gFW^{-1}, (4) calculation of normalized peak area·gFW^{-1}, (5) semi-quantification based on internal standard, (6) semi-quantification based on external calibrator area using the NearestRT *n*-alkane and (7) percentage calculation.

Method 1. Calibration curves obtained by adding standards to the sampling system

We used two different methods to apply standards to the sampling system, method 1A generates calibration curves by standard addition, whereas method 1B generates external calibration curves.

In the first case (method 1A), a mixture of standards (25, 50 and 100 mg/L) including ocimene, acetophenone, methyl benzoate and methyl cinnamate was added directly to the sucrose solution together with four individual *Antirrhinum* flowers of the same plant, distributed in four desiccators. This experiment was duplicated.

In the second case (method 1B), standards with different concentrations of ocimene, acetophenone, methyl benzoate and methyl cinnamate were added directly to the sucrose solution without flower (Table 2). The concentration ranged from 11.25 to 900 mg/L (ocimene) and from 50 to 1000 mg/L (acetophenone, methyl benzoate and methyl cinnamante). A total of 6 standard mixtures with 3 replicas were applied to different sampling systems. Calibration curves were obtained by using Chemstation. We used the total integrated peak area of each compound for further semi-quantification and the calibrator approaches A) and B) as described below.

Method 2. External calibration curves obtained by adding standards to stir bars

The same aforementioned standards were used in order to obtain calibration curves by adding liquid aliquots directly to stir bars. The concentration of ocimene ranged

Table 2 External calibration curves carried out in headspace and by liquid addition of standards to stir bars

External calibration curve	Standard	Retention time	Calibration curve	r^2	Unit
Standards to sampling system (1B)	Ocimene (E)	6.60	$6.299 \cdot 10^8 \times$	0.98	mg
	Ocimene (Z)	6.80	$1.196 \cdot 10^9 \times$	0.98	
	Acetophenone	7.20	$5.247 \cdot 10^8 \times$	0.96	
	Methyl benzoate	7.77	$1.345 \cdot 10^9 \times$	0.96	
	Methyl cinnamate	12.61	$2.891 \cdot 10^9 \times$	0.99	
Standards to stir bars (2)	Ocimene (E)	6.54	$3.424 \cdot 10^6 \times - 9.325 \cdot 10^4$	0.99	µg
	Ocimene (Z)	6.81	$8.318 \cdot 10^6 \times - 1.397 \cdot 10^5$	0.99	
	Acetophenone	7.18	$1.052 \cdot 10^7 \times - 2.693 \cdot 10^5$	0.99	
	Methyl benzoate	7.78	$1.181 \cdot 10^7 \times - 1.009 \cdot 10^5$	0.99	
	Methyl cinnamate	12.66	$1.762 \cdot 10^7 \times - 5.245 \cdot 10^5$	1	

from 25 to 500 mg/L while acetophenone, methyl benzoate and methyl cinnamate ranged from 50 to 500 mg/L. A total of 5 standard mixtures with 3 replicas were applied to different stir bars in an injection volume of 0.5 µl. Calibration curves were obtained by using Chemstation (Table 2).

As in case of method 1B, we used the total integrated peak area of each compound for furthersemi-quantification, and the calibrator approaches (A) and (B) as described below:

(A) NearestRT: semi-quantifying those compounds lacking standards by using as calibration curve the nearest component among ocimene, methyl benzoate and acetophenone. In this case for instance, we have semi-quantified nonanal (RT 7.958 min) with the methyl benzoate (RT 7.773) calibration curve.

(B) Single calibrator: using a single calibration curve (ocimene, methyl benzoate or acetophenone) for quantifying all the compounds on the scent profile of *A. majus*. For instance, using methyl benzoate calibration curve to semi-quantify the emission of each compound of interest: methyl 2-methylbutanoate, β-myrcene, ocimene, acetophenone, methyl benzoate, nonanal and acetophenone 2-hydroxy.

Method 3. Calculation of peak area per fresh weight

The relative abundance of compounds was expressed as the total integrated area of each compound divided by the FW of the sample.

Method 4. Calculation of normalized peak area per fresh weight

Normalization of peak areas of each compound was done by using 1-phenylethanol (RT 7.096, Sigma-Aldrich, P13800) as an internal standard by adding 10 µL (0.1%) to the sucrose solution during the flower scent analysis. The normalized peak areas of all compounds were calculated by dividing their total integrated peak area by the integrated peak area of the internal standard.

Method 5. Semi-quantification based on a single internal standard peak

Semi-quantification was done by extrapolating the area of 10 µL (0.1%) of 1-phenylethanol (added to each sample) to the integrated area of every compound in the profile.

Method 6. Semi-quantification based on the NearestRT n-alkane

We added 1 µl of *n*-alkane standard solution C8-C20 (Sigma Aldrich, 04070) to a stir bar. The default concentration of each *n*-alkane in the solution was 40 mg/L. Each *n*-alkane was used as an external calibrator of the NearestRT compound of interest (Table 3). Integrated areas of the NearestRT *n*-alkane were used for semi-quantification by extrapolating the area of 40 mg/L of the nearest *n*-alkane to the integrated area of the compound of interest.

Method 7. Percentage analysis

In order to calculate the percentage of VOCs from raw data profiles, we selected 7 compounds emitted both during day and night by *Antirrhinum* flowers. The sum of emission of these 7 compounds was considered as 100%.

Statistics

We performed statistical analysis on the raw data for every semi-quantification method described above.

Table 3 Alkanes used for semi-quantifying compounds of interest

RT compound	Compound	RT alkane	Alkane used for semi-quantification
2.23	Methyl 2-methylbutanoate	2.325	Octane
5.58	β-myrcene	5.820	Decane
6.62	Ocimene	5.820	Decane
7.19	Acetophenone	7.954	Undecane
7.77	Methyl benzoate	7.954	Undecane
7.96	Nonanal	7.954	Undecane
9.03	Acetophenone, 2′-hydroxy	9.797	Dodecane

Raw data generated during the day were used to analyse proportional variations of each compound within the scent profile. Second, we retrieved the differences in the day vs. night emission of the compounds of interest. Levene's, one-way ANOVA and Tukey's tests were performed by using R (Rcmdr package).

Results

Comparison of stir bars and sampling time in HSSE

We tested two types of stir bars commercially available, the PDMS and dual-phase. Based on the adsorption characteristics, dual-phase should perform better than PDMS adapted to HSSE. However, we obtained a very high background noise by using the dual-phase stir bars (Fig. 2), that may completely cover true signals from biological tissues. Our results indicate that dual-phase stir bars are not suitable to sample complex matrices, such as flowers, due to the high noisy background introduced (Fig. 2). We therefore performed the rest of the measurements and experiments with PDMS bars.

Most plants emit their floral scent preferentially at certain times of the day. Plants emitting mostly during the day include rose, narcissus or *Antirrhinum*, while plants with preferential night emission include *Petunia* or *Nicotiana* [11, 31, 32]. As sampling frequency plays a role in the detection of circadian rhythms, we tested the effect of sampling time on VOC profiles analysed by HSSE. We sampled a Petunia flower for 4 and 24 h. Figure 3 shows a chromatogram after 4 h of sampling compared to 24 h of sampling under identical conditions. Sampling periods did not seem to affect greatly the acquisition of major compound (Fig. 3a, b). However, the total number of compounds as well as abundances of VOCs were notably affected by sampling time (Fig. 3c, d). Minor VOCs such as benzyl acetate (CAS 140-11-4, RT 9.087, quality 97), benzyl 2-methylbutyrate (CAS 56423-40-6, RT 12.625, quality 97) and (Z)-isoeugenol (CAS 5912-86-7, RT 12.939, quality 98) were detected only in samplings of 24 h. Our results show that sampling time needs to be taken into account when characterising VOC profiles of plant species based on HSSE.

Comparison of semi-quantification methods

Method 1. Calibration curves obtained by adding standards to the sampling system

The feasibility of using calibration curves in the headspace was tested using two approaches. In case of method 1A, we intended to quantify four compounds, ocimene, methyl benzoate, acetophenone and methyl cinnamate by adding mixtures of these standards together with the flower. We used flowers of the same plant for each concentration in order to obtain the corresponding calibration curves and eliminate the matrix effect (Fig. 4a–e). We expected a linear evolution of peak areas for each compound, but instead we observed high variation

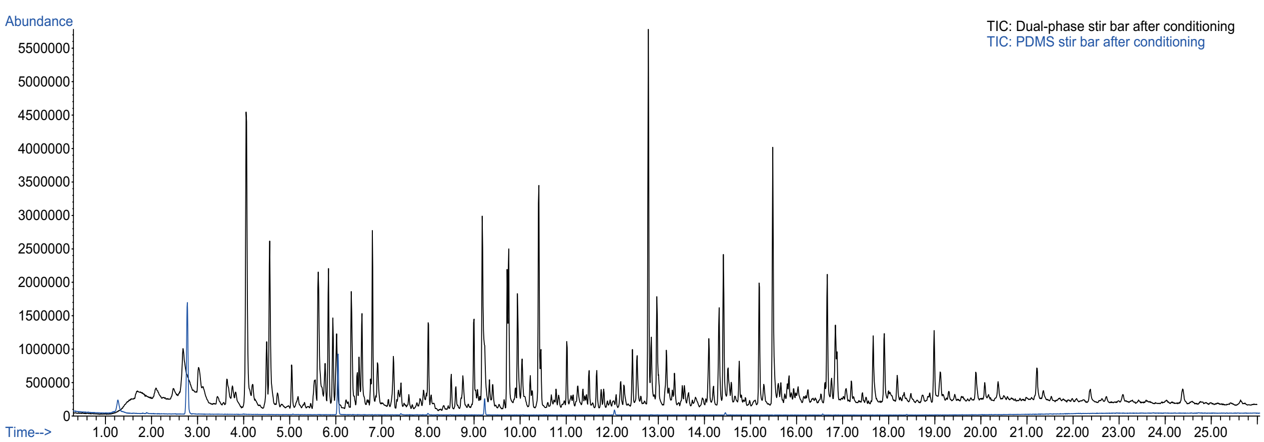

Fig. 2 Comparison of commercially available stir bars: blank chromatograms of dual-phase and PDMS stir bars after conditionings according to the indications of manufacturer

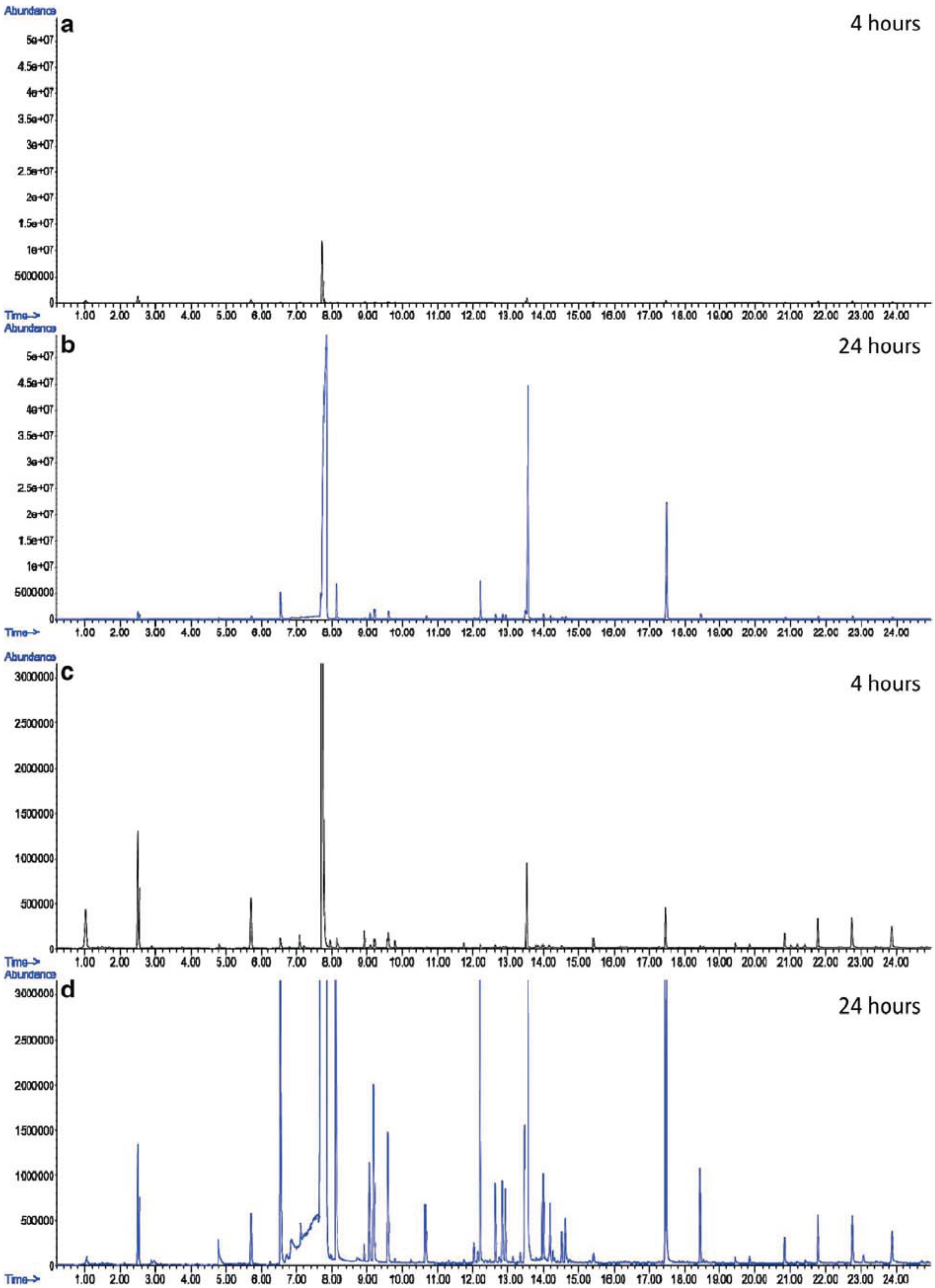

Fig. 3 Effect of sampling time on the identification of VOCs. Comparison of floral scent chromatograms of Petunia after 4 h (**a**, **c**) and 24 h (**b**, **d**) of sampling. **c**, **d** are close ups of chromatograms (**a**, **b**). Scales of **a**, **b**, and **c**, **d** are the same for comparison

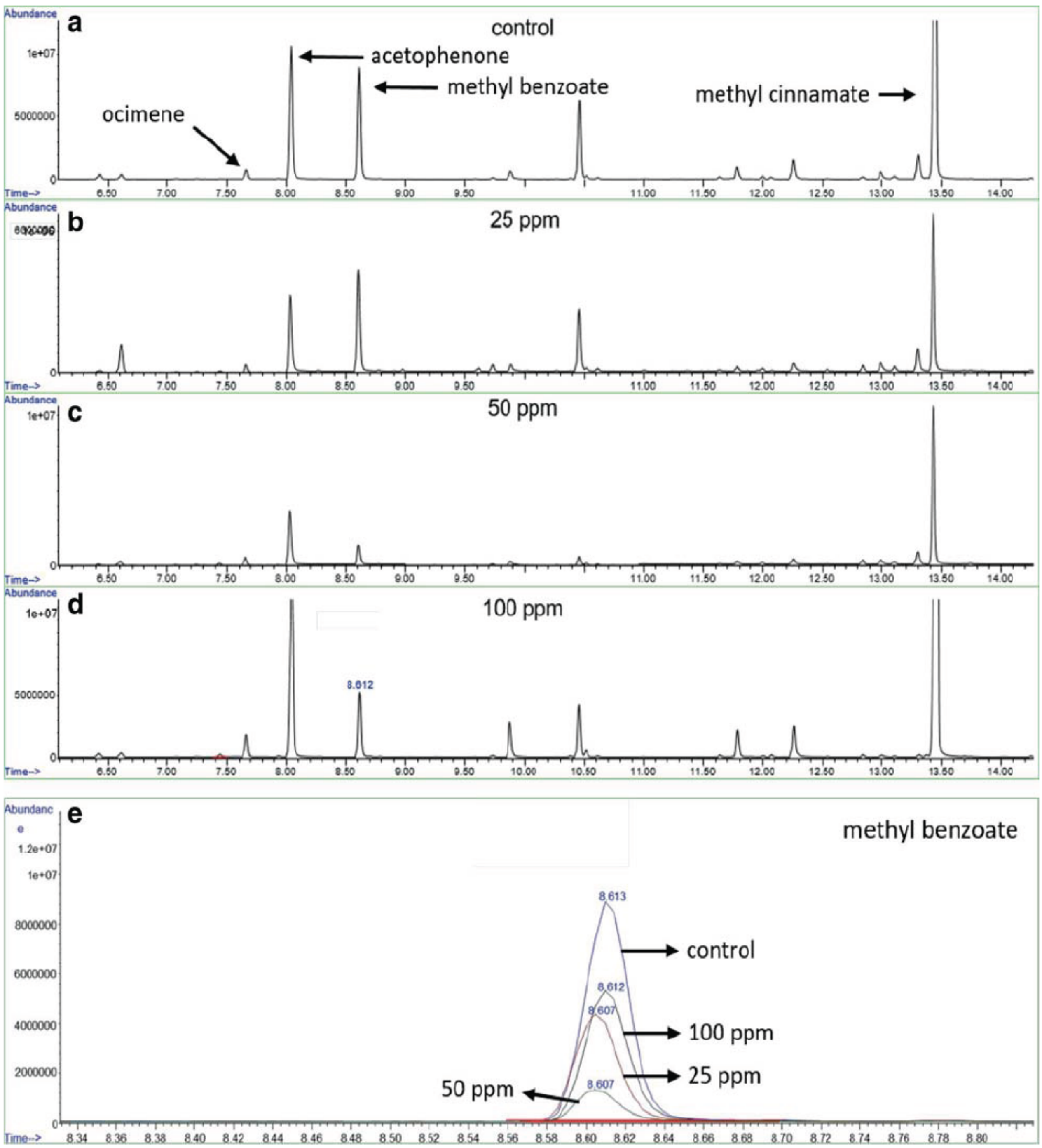

Fig. 4 VOC chromatograms of *A. majus* flowers after adding standards to the headspace. **a** Chromatogram of a control flower. **b–d** chromatograms after adding 25, 50 and 100 mg/L of the standards. The standards ocimene, acetophenone, methyl benzoate and methyl cinnamate were added directly to the headspace containing flowers from the same plant at the same developmental stages. **e** Overlaid view of the methyl benzoate peaks from chromatograms (**a–d**) (RT 8.6)

between flowers, indicating that this calibration procedure is not applicable based on the high natural variability among flowers of one plant. This problematic is illustrated in the chromatogram of a control flower that emits more methyl benzoate than those supplemented with standard solutions (Fig. 4e), indicating that this method does not allow an appropriate quantification.

In case of method 1B, we semi-quantified our raw data of *A. majus* VOCs with external calibration curves carried out by adding standards to the sampling system without flowers (Table 2) using the approaches NearestRT (semi-quantifying those compounds lacking standards by using the calibration curve of the nearest component) and single calibrator. Results show that quantities obtained by the NearestRT procedure or by a single calibrator ocimene or methyl benzoate (Fig. 5a, b, d) peaked at over 1000 μg gFW^{-1}. In contrast, in case of acetophenone as single calibrator, quantities were over a 40% higher (Fig. 5c). Concerning differences in the scent profile during the light period, we found two patterns. While single calibrators (Fig. 5b–d) resulted in an identical statistical difference pattern with significant differences among acetophenone and ocimene (Tukey p value 0.0017), acetophenone was not different from ocimene (Tukey p value 1) in case of the NearestRT calibration (Fig. 5a, Additional file 1: Tables S1, S2 and S3). Differences in VOCs between day and night using different calibrator approaches were identical with ANOVA p value of 0.0057, 0.024 and 0.017, respectively, for β-myrcene, ocimene and nonanal (Fig. 5, Additional file 1: Tables S4 and S5).

Method 2. External calibration curves carried out by liquid addition to stir bars

Following the same key principles applied to the prior way of semi-quantifying (NearestRT and single calibrator), we semi-quantified our raw data of *A. majus* VOCs emitted during the light and dark periods by using external calibration curves obtained by adding standards directly to stir bars (Table 2). We first analysed statistical

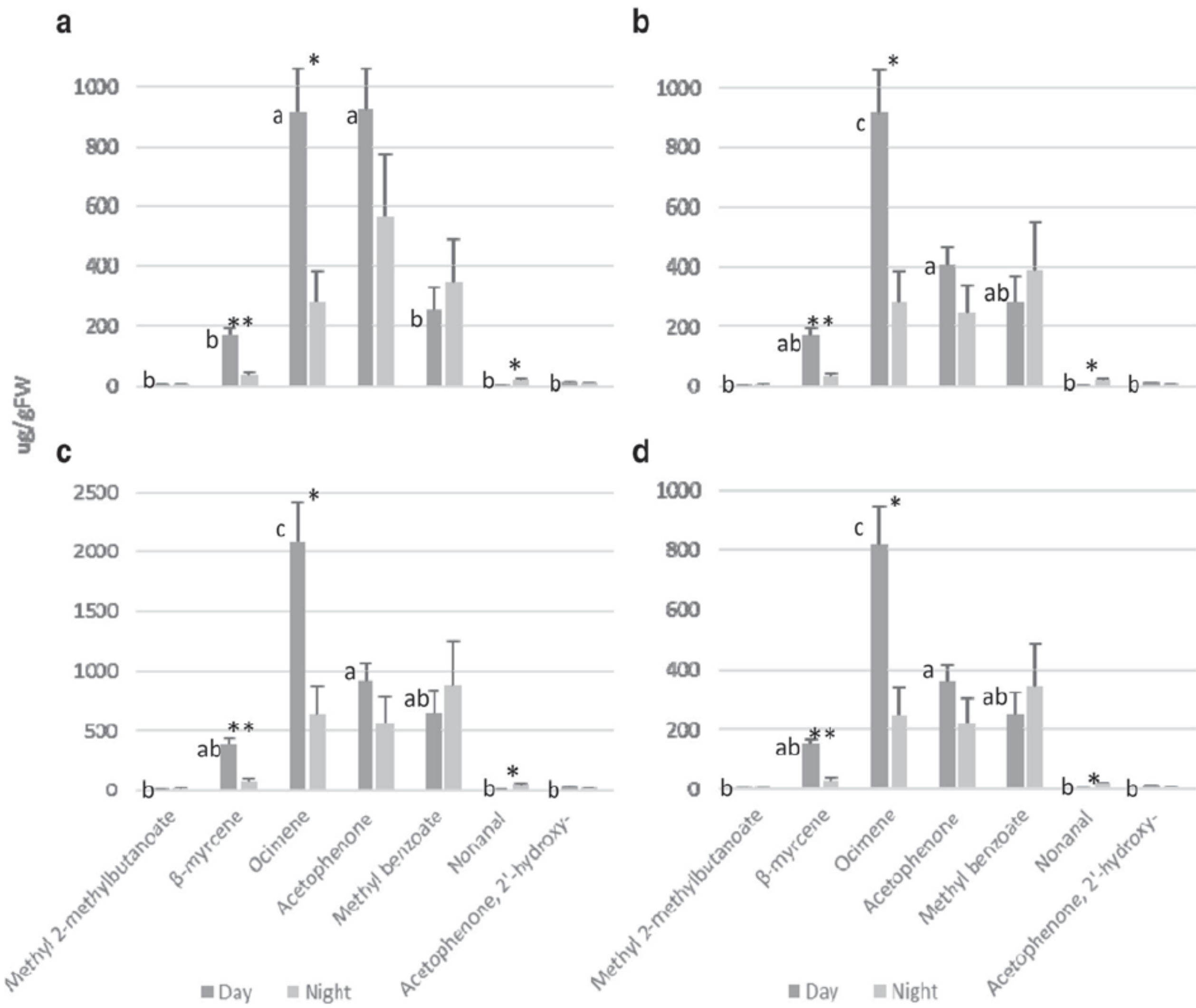

Fig. 5 Semi-quantification of VOC compounds emitted by *A. majus flowers* via external calibration curves carried out in the sampling system without flowers. **a** Application of the NearestRT calibration curve. **b** Application of single calibrator ocimene. **c** Application of single calibrator acetophenone. **d** Application of single calibrator methyl benzoate. Semi-quantifications were applied to raw data from day and night. Figures show the mean values in μg gFW^{-1} of three samples for each compound and error bars indicate the standard error. Different letters indicate statistical differences between compounds during the day. Asterisks indicate statistical differences of individual compound between day and night

differences in VOC semi-quantification during a collection period of 12 h (day) (Fig. 6, Additional file 1: Tables S1, S2 and S3). Our results indicate that total quantities of compounds varied between the different approaches. For instance, ocimene emission during the day ranged from 93 to 132 µg gFW^{-1} depending on the calibration system (Fig. 6a, b). On the other hand, the variance and the statistical significances between compounds were maintained (Fig. 5a–d) as between ocimene and acetophenone with *p* value of 0.00016 in case of the NearestRT and 0.0017 in case of the single calibrators (Tukey's test). When analysing the differences in VOC profiles between day and night, statistical significant patterns were maintained among calibrator approaches with ANOVA *p* value for β-myrcene, ocimene and nonanal (Fig. 6, Additional file 1: Tables S4 and S6) of 0.0057, 0.024 and 0.017, respectively.

Our main conclusion regarding external calibration curves is that using a single calibrator provides identical statistical results irrespective of the compound or external calibrator chosen. In contrast, using the NearestRT, may be subject to changes depending on selected compounds.

Method 3. Peak area per gram of FW

An alternative method of reporting the relative abundance of compounds is expressing the total integrated area of each compound divided by the FW of the sample

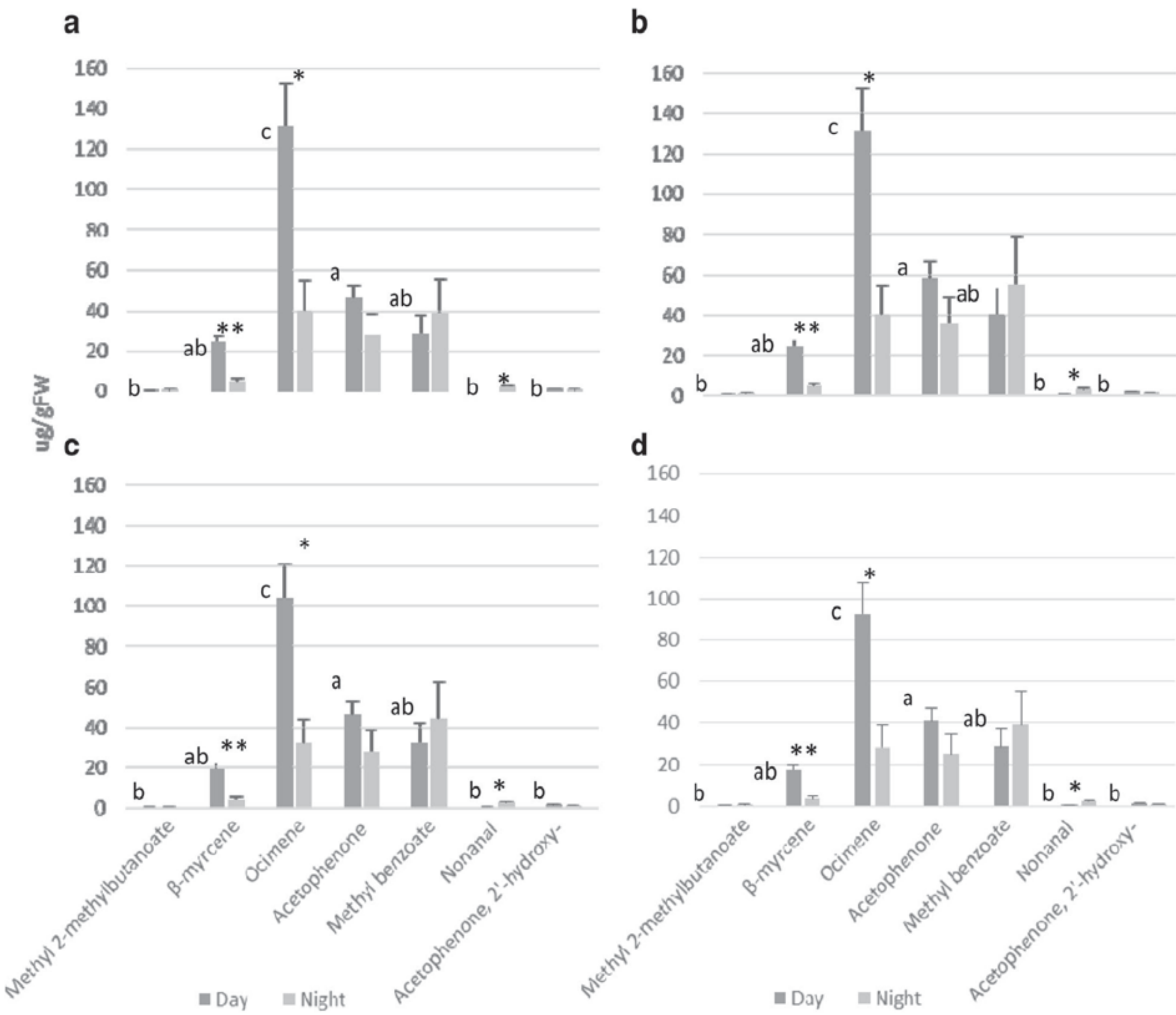

Fig. 6 Semi-quantification of VOC compounds emitted by *A. majus flowers* via external calibration curves obtained by adding standards to stir bars. **a** Application of the NearestRT calibration curve. **b** Application of single calibrator ocimene. **c** Application of single calibrator acetophenone. **d** Application of single calibrator methyl benzoate. Semi-quantifications were applied to raw data from day and night. Figures show the mean values in µg gFW^{-1} of three samples for each compound and error bars indicate the standard error. Different letters indicate statistical differences between compounds during the day. Asterisks indicate statistical differences of individual compound between day and night

(Fig. 7a). We evaluated the statistical difference pattern among the relative amounts of compounds of the daylight profile and did not find differences compared to the previously presented methods. For instance, the difference in emission between ocimene and acetophenone was significant with a Tukey's *p* value of 0.0017, identical to the value obtained by single calibrators (Additional file 1: Tables S1, S2 and S3). Similarly, when analysing the emission of each compound during day and night, statistical significances were identical to those found for the two semi-quantification approaches based on calibration curves. ANOVA *p* value for the differences in emission of β-myrcene, ocimene and nonanal during day and night were: 0.0057, 0.024 and 0.017, respectively (Additional file 1: Tables S4 and S7).

Method 4. Normalized peak area per gram of FW

Normalizing gas-chromatographic data is understood as a way of decreasing the experimental error. For this normalization procedure, it is necessary to add a certain quantity of an internal standard not emitted by the sample.

Concerning the scent profiles emitted during the day, statistical results varied slightly compared to the methods applying the total integrated peak areas (see above). According to Tukey's test, acetophenone and ocimene emission were not statistically different (*P* 0.056) (Fig. 7b, Additional file 1: Tables S1, S2 and S3). Likewise, comparative analysis of the emission between day and night resulted in a different statistical result compared to the previously described semi-quantitative approaches. In this case, the emission of β-myrcene and ocimene were significantly different (*P* 0.0022 and 0.0092), but nonanal was not (*P* 0.072) (Fig. 7b, Additional file 1: Tables S4 and S7).

Method 5. Semi-quantification based on internal standard abundance

A quite extended method of semi-quantifying the emission of compounds is to extrapolate the integrated area of the added internal standard to the integrated area of any compound in the chromatogram.

When applying this method, mean quantities of every compound in the scent profile increased considerably compared to the semi-quantitative methods based on calibration curves. Regarding the statistical difference pattern among scent compounds emitted during the day as well as between day and night, results were identical to the normalized peak area method. During the day, a statistical difference between acetophenone and ocimene emission was lacking (Tukey *p* value 0.056), (Fig. 7c, Additional file 1: Tables S1, S2 and S3). Similarly between day and night, the emission of β-myrcene and ocimene (*P* 0.0022 and 0.0092) was statistically significant, but not of nonanal (*P* 0.072) (Fig. 7c, Additional file 1: Tables S4 and S7).

Method 6. Semi-quantification based on the nearest n-alkane

Following the key principle that similar compounds elute at similar retention times, we used *n*-alkanes to semi-quantify our raw data. The nearest in retention time *n*-alkane (NearestRT alkane) has been used to semi-quantify the compounds of interest. As an example, β-myrcene and ocimene (RT 5.57 and 6.615 min, respectively) have been semi-quantitated by using decane (RT 5.82 min) abundance (Table 3).

The analysis of the scent profile during the day (Fig. 7d, Additional file 1: Tables S1, S2 and S3) indicates that quantities of compounds varied largely when compared to the rest of approaches studied. Regarding the statistical difference pattern among compounds, these differences were similar to those obtained in semi-quantifications based on the methods using total peak areas (Fig. 7d), with a statistical difference between acetophenone and ocimene of *p* value 0.025 (Tukey). Similarly, differences between day and night emission of β-myrcene, ocimene and nonanal were statistically significant (ANOVA *p* value 0.0058, 0.024 and 0.018, respectively) as already observed for the methods based on total peak areas (Fig. 7d, Additional file 1: Tables S4 and S7).

Method 7. Percentage analysis

A convenient way to show the relative abundance of VOCs within a scent profile is to express the data in percentages. We found that the statistical significance pattern among the compounds emitted during the day was similar to the NearestRT *n*-alkane method as well as those based on total peak area. We found a statistical difference between acetophenone and ocimene of Tukey´s *p* value 0.00062 (Fig. 7e, Additional file 1: Tables S1, S2 and S3). Concerning statistical differences between day and night, β-myrcene and nonanal showed significant difference whereas ocimene did not. ANOVA *p* value for β-myrcene, ocimene and nonanal were: 0.035, 0.132 and 0.0043, respectively (Fig. 7e, Additional file 1: Tables S4 and S7).

Discussion

Most studies on VOCs emission concentrate on one to several compounds emitted by a set of samples [33–36], whereas fewer studies focus on entire scent profiles [8, 37–39]. The reason lies in the complexity of this type of analyses. Firstly, it requires the selection of an appropriate chromatographic method which allows to detect all emitted VOCs [18, 40]. Secondly, many variables influence the quantity of VOCs released from the samples,

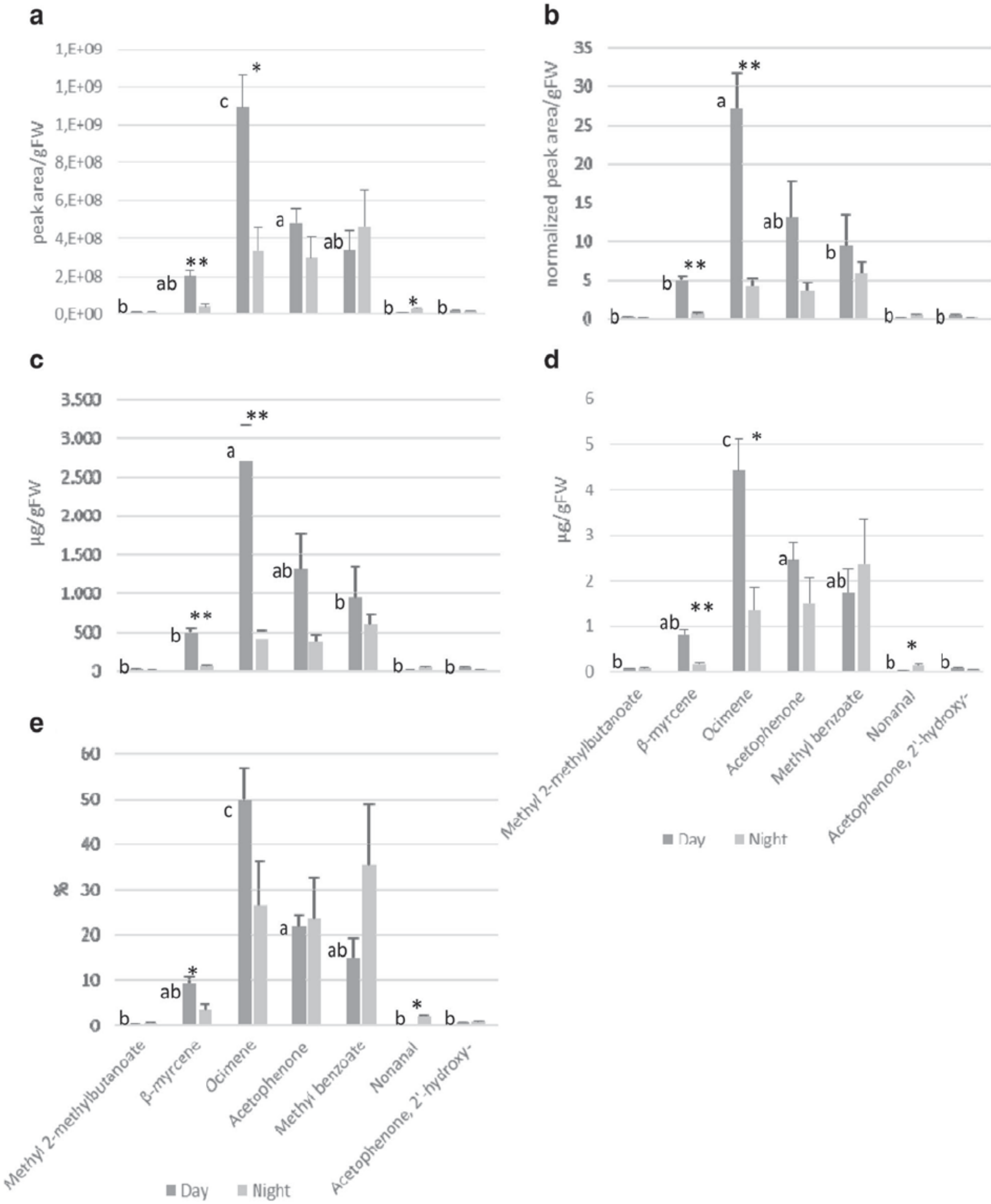

Fig. 7 Semi-quantifications of *A. majus* flowers profiles by using different methodologies based on: **a** total integrated peak area **b** normalized area, **c** single internal standard peak (1-phenylethanol), **d** NearestRT *n*-alkane abundance and **e** percentage abundance. Semi-quantifications were applied to raw data from day and night. Figures show the mean values of three samples for each compound and error bars indicate the standard error. Different letters indicate statistical differences between compounds during the day. Asterisks indicate statistical differences of individual compound between day and night

such as light, temperature [37], physiological status of tissues [34] and even air pollution [41], leading to a high variability in the collected data [16]. Many investigators therefore concentrate on a few major compounds, which are constitutively emitted by their research objects, rather than dealing with minor compounds that may or may not be found due to reasons such as natural variability, VOC contamination or VOC emission in a circadian fashion [42]. On the other hand, studying the entire scent profile may give insight in very complex phenomena such as interactions between plants and pollinators [40, 43, 44], volatile perception related to disease detection [7, 45–47], or pheromone signalling [48, 49]. Aromas are phenotypic traits that identify species as a result of evolutionary selection. Establishing which compounds and in which proportion contribute to scent profiles, is a determinant issue for characterizing species as well as for evaluating their effects over different taxa [5, 8, 40, 50].

Sampling time and stir bars for HSSE

The use of stir bars in HSSE analysis requires an atmosphere in equilibrium, sealed and isolated. PDMS coated stir bars preferably adsorb non-polar compounds. VOCs with different volatility will therefore be adsorbed differentially according to their chemical features [22, 51]. As shown in this work, the number of compounds identified is directly related to the time samples are exposed to the stir bars. This has important implications as VOCs emissions are under circadian regulation in many plants including *Antirrhinum* and *Petunia* [11, 32]. As sampling time plays a key role in the detection of rhythms, and sampling time of 4 h is the minimum required [25], our results indicate that increasing the sampling density will necessarily result in fewer minor VOCs identified.

Additionally, stir bars in headspace show a very high relative standard deviation [52]. Variability using stir bars results from two factors: changes in temperature and the matrix effect. The matrix effect is caused by the equilibrium conditions between matrix/headspace and the headspace/PDMS of the stir bar [52, 53]. In our study, temperature was under strict control and its effects on variability can be excluded. However, the matrix effect is difficult to control when complex matrices are used, such as plant tissues or organs.

Normalized area vs total peak area

Semi-quantification of compounds using normalized peak area is based on the usage of a specific amount of an internal standard. However, the standard may have chemical characteristics different to the compounds emitted by the sample and therefore may be adsorbed differently by the stir bar. This may cause miscalculations of quantities of VOCs emitted by samples. The use of dual-phase stir bars may improve detection of volatiles, because they more effectively recover polar analytes [54]. However, some drawbacks have been reported [44] and the degree of noise background introduced by these stir bars impedes the identification of minor VOCs.

Comparison of NearestRT approaches

Based on the key principle that chemically similar compounds elute at similar retention times, we used calibrators of compounds commonly found in the scent profile of *A. majus* as well as *n*-alkanes, which elute at similar retention times as the sample´s compounds, for semi-quantification. Both VOC quantities and proportions varied among the different NearestRT approaches, independent of whether calibrators were added to the headspace or as liquid to stir bars. To our knowledge, this is the first time that *n*-alkanes have been reported for semi-quantifying VOCs as external calibrators. While the NearestRT approach has been applied in combination with internal standards [15, 21], its usage with external calibrators is not documented. We show that the NearestRT approaches presented here are equally valuable for scent profiling.

Advantages and disadvantages of the studied methodologies

We compared the effect of semi-quantification methods within scent profiles (day time samples) and between scent profiles (day and night time samples). The volatile proportions obtained by different semi-quantification methods, both on intra-sample and inter-sample data, even so not being identical clearly showed a similar trend. These small proportional discrepancies caused, in some cases, statistical variations. Nevertheless we consider that these small variations do not compromise any of the methods studied.

As shown in this work, several valid approaches exist for analysing GC–MS data using HSSE and TICs in terms of semi-quantification. All these procedures provide general, yet accurate, information about profile features. Nevertheless, each of the methodologies analysed here beholds specific advantages and disadvantages related to accuracy, experimental variability, acceptance and retrieved quantities (Table 4).

Regarding accuracy, the use of the NearestRT approach (methods 1B, 2 and 6) could be considered more appropriate than the single calibrator approach (methods 1B and 2) and internal standard abundance (method 5), because chemically more similar compounds are used.

Concerning experimental variability, the outcome of external calibration curves in headspace is affected by the experimental conditions, like sampling time or volume in the headspace container, and need to be adapted to those

Table 4 Overview of advantages and disadvantages of the semi-quantifying approaches

Advantages	Disadvantages
Method 1B. (*) External calibration curves obtained by adding standards to the sampling system	
High accuracy due to identical sampling conditions between external calibrators and samples	Calibration curves are valid only for the specific sampling conditions (i.e. time or headspace volume)
Method 2. (*) External calibration curves obtained by adding standards to stir bars	
Calibration curves are valid independent of sampling conditions	Lower accuracy due to different sampling conditions between external calibrators and samples
*NearestRT	
High accuracy due to the usage of chemically similar compounds for semi-quantification	Several calibrators along the chromatogram need to be used
*Single calibrator	
Statistical significance of the data is consistent, indicating that any calibrator is valid	A certain level of inaccuracy may result from a lack of chemical similarity between calibrator and sample VOCs
Method 3. Peak area/g fresh weight	
It indicates the relative abundance among VOCs	There is no magnitude
Method 4. Normalized peak area/g fresh weight	
Generally accepted as a precise mean to analyze relative abundance among VOCs	Bias due to differential stir bar adsorption between the internal standard and certain kinds of VOCs There is no magnitude
Method 5. Single internal standard peak	
Generally accepted as a precise mean for semi-quantification	Bias due to differential stir bar adsorption between the internal standard and certain kinds of VOCs
Method 6. NearestRT n-alkane	
High accuracy due to the usage of chemically similar compounds for semi-quantification	A certain level of inaccuracy may result from a lack of chemical similarity between calibrator and sample VOCs
Method 7. Percentage analysis	
Generally accepted as a precise mean to analyze relative abundance among VOCs	There is no magnitude

The asterisks indicate that NearestRT and single calibrator were used in method 1B and method 2

used for VOC sampling. In contrast, this problematic does not occur when applying methods based on liquid addition directly to stir bars (method 2 and 6).

Quite popular methodologies are those based on internal standard abundance [10, 19, 20, 40] and percentages [14, 31, 55, 56], because they accurately reflect relative abundances. We show here that less common methods like total peak areas or normalized areas of compounds [8, 14] are valid alternatives for this purpose. However, none of these two approaches have a magnitude and understanding abundances is not straightforward. The single calibrator approach has been reported, but with some methodological differences [57] and no publications are available expressing results as total peak areas·gFW^{-1}.

Regarding VOC quantities, the only informative methods are: external calibration curves from headspace (method 1B), external calibration curves from liquid addition to stir bars (method 2), semi-quantification based on a single internal standard peak (method 5) and semi-quantification based on the NearestRT *n*-alkane. However, quantities may vary depending on the methods and this difference should be taken into account especially when comparing results from different publications.

Despite all the divergences, all methods can be considered as reliable means of analysing scent profiles.

Conclusions

Two main conclusions can be drawn from this study. First, semi-quantification by standard addition is not a feasible method in sets of samples with a high biological variability, as in case of flowers. Secondly, any of the methodologies studied adequately reflects the relative proportion of VOCs when screening volatile metabolomes.

From our point of view and concerning the plant scent community, a general methodological consensus would be desirable in order to ease the comparison of data.

Additional file

Additional file 1. Statistical analysis of different semiquantitative methods.

Authors' contributions
VRH and MJR conceived the ideas and designed methodology; VRH, MJR, MEC and JW collected the data; VRH and MJR analysed the data; VRH, MJR, MEC and JW wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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Supplementary material

Table S1. Levene´s test for homoscedasticity of all *A. majus* flowers VOCs emitted during the day, applying the different semi-quantification methods.

Method (nr.)	F value	P-value
Single calibrator: standards to sampling system (1B) and to stir bars (2); total peak area·g FW (3)	1.017	0.4534
NearestRT standards to sampling system (1B)	1.0642	0.428
NearestRT standards to stir bars (2)	1.1201	0.3996
Normalized peak area·g FW (4); single internal standard peak (5)	1.6699	0.2009
NearestRT *n*-alkane (6)	0.9522	0.4903
Percentage (7)	1.572	0.2271

P values ≤ 0,05 are considered significant. Degrees of freedom: 6.

Table S2. Statistic significance among VOCs for the entire day-time scent profile of *A. majus* flowers according to ANOVA.

Method (nr)	Calibration curve	Sum Sq	Mean Sq	F-value	P-value	Signif.
Standards to sampling system (1B)	NearestRT	3105655	517609	25.57	0.000000884	***
	Ocimene	1947909	324651	23.15	0.00000165	***
	Acetophenone	10120660	1686777	23.15	0.00000165	***
	Methyl benzoate	1540233	256705	23.15	0.00000165	***
Standards to stir bars (2)	NearestRT	39212	6535	26.8	0.000000658	***
	Ocimene	40271	6712	23.15	0.00000165	***
	Acetophenone	25177	4196	23.15	0.00000165	***
	Methyl benzoate	19977	3330	23.15	0.00000165	***
Total peak area·g FW (3)		2,786E+21	4,644E+20	23.15	0.00000165	***
Normalized peak area·g FW (4)		1749.9	291.65	11.17	0.000119	***
Single internal standard peak (5)		17498702	2916450	11.17	0.000119	***
Nearest *n*-alkane (6)		5.50	8.031	20.43	0.00000354	***
Percentage (7)		5707	951.1	28.25	0.000000472	***

*,**, and *** indicate statistical significances for p values ≤ 0.05, ≤ 0.01 and 0.001, respectively and 6 degrees of freedom.

Table S3. Statistical significance among specific VOCs of the day-time scent profile of *A. majus* flowers according to TukeyHSD test. Statistical significance p-value < 0.05. Bold numbers indicate statistical significances.

Compounds	Single calibrators: samp. syst. (1B) and stir bars (2); total peak area·g FW (3)	Headspace NearestRT (1B)	Liquid addition NearestRT (2)	Normalized peak area·g FW (4); single internal standard peak (5)	NearestRT *n*-alkane (6)	Percent (7)
Acetophenone, 2-hydroxy--Acetophenone	**1,62E-02**	**2,91E-05**	**4,34E-02**	9,54E-02	**7,93E-03**	**5,17E-03**
Methyl 2-methylbutanoate-Acetophenone	**1,40E-02**	**2,69E-05**	**4,02E-02**	8,63E-02	**6,76E-03**	**5,06E-03**
Methyl benzoate-Acetophenone	8,63E-01	**7,60E-04**	8,16E-01	9,69E-01	7,88E-01	7,74E-01
Nonanal-Acetophenone	**1,34E-02**	**2,61E-05**	**3,77E-02**	8,37E-02	**6,46E-03**	**4,34E-03**
Ocimene-isomers-Acetophenone	**1,72E-03**	1,00E+00	**1,56E-04**	5,61E-02	**6,16E-04**	**2,45E-02**
ß-myrcene-Acetophenone	2,62E-01	**2,37E-04**	6,38E-01	4,80E-01	1,90E-01	7,29E-02
Methyl 2-methylbutanoate-Acetophenone, 2-hydroxy	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00
Methyl benzoate-Acetophenone, 2-hydroxy-	1,47E-01	4,22E-01	3,82E-01	3,74E-01	9,96E-02	6,95E-02
Nonanal-Acetophenone, 2-hydroxy-	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00
Ocimene-isomers-Acetophenone, 2-hydroxy-	**3,70E-06**	**3,12E-05**	**1,20E-06**	**2,62E-04**	**1,10E-06**	**1,15E-05**
ß-myrcene-Acetophenone, 2-hydroxy-	6,71E-01	8,11E-01	5,60E-01	9,21E-01	5,65E-01	7,59E-01
Methyl benzoate-Methyl 2-methylbutanoate	1,29E-01	3,94E-01	3,62E-01	3,46E-01	8,57E-02	6,82E-02
Nonanal-Methyl 2-methylbutanoate	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00
Ocimene-isomers-Methyl 2-methylbutanoate	**3,30E-06**	**2,88E-05**	**1,20E-06**	**2,39E-04**	**1,00E-06**	**1,13E-05**
ß-myrcene-Methyl 2-methylbutanoate	6,25E-01	7,85E-01	5,36E-01	9,02E-01	5,15E-01	7,53E-01
Nonanal-Methyl benzoate	1,24E-01	3,83E-01	3,44E-01	3,38E-01	8,20E-02	5,86E-02
Ocimene-isomers-Methyl benzoate	**2,09E-04**	**8,23E-04**	**2,03E-05**	**1,13E-02**	**6,40E-05**	**1,83E-03**
ß-myrcene-Methyl benzoate	8,96E-01	9,90E-01	1,00E+00	9,28E-01	8,78E-01	5,91E-01

Ocimene-isomers-Nonanal	**3,20E-06**	**2,79E-05**	**1,10E-06**	**2,32E-04**	**9,00E-07**	**1,00E-05**
ß-myrcene-Nonanal	6,12E-01	7,73E-01	5,15E-01	8,95E-01	5,00E-01	7,06E-01
ß-myrcene-Ocimene-isomers	**3,47E-05**	**2,56E-04**	**1,28E-05**	**1,66E-03**	**1,10E-05**	**9,85E-05**

Statistical significance p-value < 0.05.

Table S4. Levene´s test for homoscedasticity of all *A. majus* flower VOCs emitted during day and night, applying the different semi-quantification methods.

Method (nr)	Compound	F-value	P-value
External calibration curves obtained by adding standards to sampling system (1B) and to stir bars (2); total peak area·g FW (3)	Methyl 2-methylbutanoate	1.2527	0.3257
	β-myrcene	0.6882	0.4534
	Ocimene-isomers	0.0681	0.8071
	Acetophenone	0.1294	0.7373
	Methyl benzoate	0.3905	0.5659
	Nonanal	3.2407	0.1462
	Acetophenone 2-hydroxy	0.2615	0.636
Normalized peak area·g FW (4); single internal standard peak (5)	Methyl 2-methylbutanoate	0.0089	0.9293
	β-myrcene	1.7235	0.2595
	Ocimene-isomers	1.2463	0.3268
	Acetophenone	1.6384	0.2697
	Methyl benzoate	1.4091	0.3009
	Nonanal	1.7789	0.2532
	Acetophenone 2-hydroxy	1.2246	0.3305
NearestRT *n*-alkane (6)	Methyl 2-methylbutanoate	1.2527	0.3257
	β-myrcene	0.6466	0.4664
	Ocimene-isomers	0.0612	0.8168
	Acetophenone	0.1377	0.7294
	Methyl benzoate	0.4265	0.5493
	Nonanal	3.4289	0.1377
	Acetophenone 2-hydroxy	0.2429	0.6479
Percentage (7)	Methyl 2-methylbutanoate	1,7377	0.2579
	β-myrcene	0.011	0.9214
	Ocimene-isomers	0.0294	0.8723
	Acetophenone	0.6028	0.4809
	Methyl benzoate	1.381	0.3051
	Nonanal	3.6833	0.1274
	Acetophenone 2-hydroxy	0.3369	0.5927

P values ≤ 0,05 are considered significant. Degrees of freedom: 1

Table S5. Statistic significance between VOCs emitted by *A. majus* flowers during the day and night according to ANOVA using the external calibration curve generated by adding standards to the headspace (Method 1B).

Calibration curve	Compound	Sum/Mean Sq	F-value	P-value	Signif.
NearestRT: Ocimene	Methyl 2-methylbutanoate	0.145	0.039	0.854	
NearestRT: Ocimene	β-myrcene	27614	29.13	0.0057	**
NearestRT: Ocimene	Ocimene-isomers	605483	12.57	0.0239	*
NearestRT: Acetophenone	Acetophenone	190105	1.974	0.233	
NearestRT: Methyl benzoate	Methyl benzoate	12952	0.321	0.601	
NearestRT: Methyl benzoate	Nonanal	438.2	15.76	0.0165	*
NearestRT: Methyl benzoate	Acetophenone 2-hydroxy	20.031	6.392	0.0648	
Single calibrator: Ocimene	Methyl 2-methylbutanoate	0.145	0.039	0.854	
	β-myrcene	27614	29.13	0.0057	**
	Ocimene-isomers	605483	12.57	0.0239	*
	Acetophenone	36589	1.974	0.233	
	Methyl benzoate	16380	0.321	0.601	
	Nonanal	554.2	15.76	0.0165	*
	Acetophenone 2-hydroxy	25.332	6.392	0.0648	
Single calibrator: Acetophenone	Methyl 2-methylbutanoate	0.75	0.039	0.854	
	β-myrcene	143472	29.13	0.0057	**
	Ocimene-isomers	3145882	12.57	0.0239	*
	Acetophenone	190105	1.974	0.233	
	Methyl benzoate	85106	0.321	0.601	
	Nonanal	2879	15.76	0.0165	*
	Acetophenone 2-hydroxy	131.62	6.392	0.0648	
Single calibrator: Methyl benzoate	Methyl 2-methylbutanoate	0.1148	0.039	0.854	
	β-myrcene	21835	29.13	0.0057	**
	Ocimene-isomers	478762	12.57	0.0239	*
	Acetophenone	28931	1.974	0.233	
	Methyl benzoate	12952	0.321	0.601	
	Nonanal	438.2	15.76	0.0165	*
	Acetophenone 2-hydroxy	20.031	6.392	0.0648	

*,**, and *** indicate statistical significances for p values ≤ 0.05, ≤ 0.01 and 0.001, respectively and 1 degree of freedom.

Table S6. Statistic significance between VOCs emitted by *A. majus* flowers during the day and night according to ANOVA using the external calibration curve generated by adding standards to stir bars (Method 2).

Calibration curve	Compound	Sum/Mean Sq	F value	P-value	Signif.
NearestRT: Ocimene	Methyl 2-methylbutanoate	0.003	0.039	0.854	
NearestRT: Ocimene	β-myrcene	570.9	29.13	0.0057	**
NearestRT: Ocimene	Ocimene-isomers	12518	12.57	0.0239	*
NearestRT: Acetophenone	Acetophenone	472.9	1.974	0.233	
NearestRT: Methyl benzoate	Methyl benzoate	168	0.321	0.601	
NearestRT: Methyl benzoate	Nonanal	5.684	15.76	0.0165	*
NearestRT: Methyl benzoate	Acetophenone 2-hydroxy	0.25980	6.392	0.0648	
Single calibrator: Ocimene	Methyl 2-methylbutanoate	0.003	0.039	0.854	
	β-myrcene	570.9	29.13	0.0057	**
	Ocimene-isomers	12518	12.57	0.0239	*
	Acetophenone	756.4	1.974	0.233	
	Methyl benzoate	338.6	0.321	0.601	
	Nonanal	11.458	15.76	0.0165	*
	Acetophenone 2-hydroxy	0.5237	6.392	0.0648	
Single calibrator: Acetophenone	Methyl 2-methylbutanoate	0.00188	0.039	0.854	
	β-myrcene	356.9	29.13	0.0057	**
	Ocimene-isomers	7826	12.57	0.0239	*
	Acetophenone	472.9	1.974	0.233	
	Methyl benzoate	211.7	0.321	0.601	
	Nonanal	7.163	15.76	0.0165	*
	Acetophenone 2-hydroxy	0.3274	6.392	0.0648	
Single calibrator: Methyl benzoate	Methyl 2-methylbutanoate	0.00149	0.039	0.854	
	β-myrcene	283.20	29.13	0.0057	**
	Ocimene-isomers	6210	12.57	0.0239	*
	Acetophenone	375.2	1.974	0.233	
	Methyl benzoate	168	0.321	0.601	
	Nonanal	5.684	15.76	0.0165	*
	Acetophenone 2-hydroxy	0.25980	6.392	0.0648	

*,**, and *** indicate statistical significances for p values ≤ 0.05, ≤ 0.01 and 0.001, respectively and 1 degree of freedom.

Table S7. Statistic significance between VOCs emitted by *A. majus* flowers during the day and night according to ANOVA using specified methods.

Method (nr.)	Compound	Sum/Mean Sq	F value	P-value	Signif.
Total peak area·gFW (3)	Methyl 2-methylbutanoate	2,0762E+11	0.039	0.854	
	β-myrcene	3,95E+19	29.13	0.0057	**
	Ocimene-isomers	8,66E+20	12.57	0.0239	*
	Acetophenone	5,23E+19	1.974	0.233	
	Methyl benzoate	2,34E+19	0.321	0.601	
	Nonanal	7,93E+17	15.76	0.0165	*
	Acetophenone 2-hydroxy	3,6236E+13	6.392	0.0648	
Normalized peak area·gFW (4)	Methyl 2-methylbutanoate	0.00563	0.565	0.494	
	β-myrcene	29.032	49.29	0.00217	**
	Ocimene-isomers	786.9	22.38	0.0091	**
	Acetophenone	132.4	3.859	0.121	
	Methyl benzoate	18.36	0.664	0.461	
	Nonanal	0.1373	5.937	0.0715	
	Acetophenone 2-hydroxy	0.09929	3.405	0.139	
Single internal standard peak (5)	Methyl 2-methylbutanoate	56.3	0.565	0.494	
	β-myrcene	290318	49.29	0.00217	**
	Ocimene-isomers	7868758	22.38	0.0091	**
	Acetophenone	1324543	3.859	0.121	
	Methyl benzoate	183556	0.664	0.461	
	Nonanal	1373	5.937	0.0715	
	Acetophenone 2-hydroxy	992.9	3.405	0.139	
Nearest *n*-alkane (6)	Methyl 2-methylbutanoate	1.92307	0.039	0.854	
	β-myrcene	64523.1079	28.99	0.00575	**
	Ocimene-isomers	1414008.04	12.5	0.0241	*
	Acetophenone	138300.863	1.968	0.233	
	Methyl benzoate	56520.1196	0.292	0.618	
	Nonanal	1943.9696	15	0.018	*
	Acetophenone 2-hydroxy	77.37176	6.916	0.0582	
Percentage (7)	Methyl 2-methylbutanoate	0.08455	4.168	0.111	
	β-myrcene	51.73	9.825	0.035	*
	Ocimene-isomers	798.5	3.555	0.132	
	Acetophenone	5.4	0.041	0.849	
	Methyl benzoate	629.8	2.14	0.217	
	Nonanal	5.291	33.98	0.00431	**
	Acetophenone 2-hydroxy	0.028531	5.29	0.0829	

*,**, and *** indicate statistical significances for p values ≤ 0.05, ≤ 0.01 and 0.001, respectively and 1 degree of freedom.

Chapter IV

gcProfileMakeR: an R Package for Automatic Identification of Constitutive and Non-Constitutive Metabolites

gcProfileMakeR: an R package for automatic identification of constitutive and non-constitutive metabolites

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Abstract

Plant metabolomes are comprised by a large number of chemicals, making non target metabolomics projects a challenge. Genetic and environmental cues affect the composition and relative quantity of different metabolites. As a result, we often find a constitutive metabolome and a set of inducible metabolites, increasing the complexity to evaluate genetic and/or environmental effects on metabolome structures. We developed gcProfileMakeR, an R package that uses standard output files from GC MS and obtains a list of core and non constitutive metabolomes, using CAS numbers. gcPRofileMakeR first defines the number and identity of the compounds present in each sample. In a second step, it determines the shared compounds for a set of samples and annotates both shared and individual compounds. The last step annotates the core metabolome (Profile) of a set of samples. It gives two additional outputs the Non Constitutive by Frequency metabolites which appear in low frequency and Non Constitutive by Quality, that have low matching to the mass spectra libraries. As a proof of concept, we defined the scent profile of *Antirrhinum majus compacta (co)* and *deficiens nicotianoides* (*def nic*) mutants. Our results show that floral organ identity genes are required to obtain floral scent profiles that can be distinguished from the green/herbal profiles found in the *def nic* mutant. gcProfileMakeR enhances the quality

of data reported in metabolomic profiles as combinations of chemical and common names are avoided and outputs rely on CAS numbers. This is especially important for FAIR data implementation.

Introduction

Plants, bacteria and animals emit complex mixtures of Volatile Organic Compounds (VOCs). Scent profiles are considered as the core volatile metabolome of a tissue, organ or organism. Developmental processes underlie the actual structure of a scent profile, as leaves, roots, flowers or fruits emit very different combinations of VOCs (Schulz Bohm et al., 2018). Furthermore, volatiles emitted in a non constitutive way may play important biological roles. The emission of non constitutive volatiles is well documented for some VOCs which are produced under abiotic stress conditions or biotic interactions (Kessler and Baldwin, 2002; Shimoda et al., 2012; Groen et al., 2016). VOCs implications in ecology and evolution have been broadly discussed and have even been identified as distinct blends that differentiate species within a genus (Knudsen et al., 2006; Schulz and Dickschat, 2007; Schiestl and Johnson, 2013; Weiss et al., 2016b).

Automatic gas chromatography mass spectrometry (GC MS) data annotation is relatively easy nowadays thanks to the development of mass spectra libraries and programs created for this purpose (Domingo Almenara et al., 2017b). Non targeted metabolomics has been proved as a valuable approach in order to identify metabolites with relevant functions in the interaction of plants and their environments (Gaquerel et al., 2009; Sánchez et al., 2013). These untargeted screenings are useful tools applied in the search of candidate genes or enzymes implicated in the synthesis of VOCs (Wei et al., 2016). The structure of a metabolome i.e. the specific metabolites that comprise a sample, and their quantities are two aspects defining metabolomics (Ricroch et al., 2011). However, reaching a consensus among samples of which compounds are comprising the constitutive metabolomics profile and which form the non constitutive metabolome or discriminate between two sets of samples is mainly done manually and criteria are not always obvious or consistent. An additional issue is the complexity of names given to a single chemical compound. In many cases, they include a common name, a chemical structure and sometimes isomers. The Chemical Abstract Service Number or CAS number is a single identifier that allows unambiguous assignation of a chemical structure. Thus the adoption of CAS number defined metabolomes is the most appropriate way to produce metabolomics raw data in a suitable format for FAIR data management (Wilkinson et al., 2016).

Here we provide an R package that uses as inputs spreadsheet files produced by GC MS apparatus to determine the core metabolome and non constitutive compounds emitted by a certain set of samples, allowing the comparison between samples by giving a set of common and differential metabolites in an automatic fashion. We demonstrate the utility of gcProfileMakeR by analysing two genotypes of *Antirrhinum majus* affecting petal identity (Manchado Rojo et al., 2012).

Materials and Methods

Plant material and VOCs analysis

We used flowers from *Antirrhinum majus compacta (co)* and *deficiens nicotianoides* (*def nic*) mutants (Manchado Rojo et al., 2012) (Figure 1). Plants were grown in the greenhouse as described previously, using standard methods (Weiss et al., 2016a). Scent samples were analysed according to Ruiz Hernández et al., 2017 and Ruiz Hernández et al., 2018. Sampling periods of VOCs were 24 hour. We analysed 29 biological replicas for co and 9 for *def nic*.

gcProfileMakeR

gcProfileMakeR R package is available at git clone git@gitlab.atica.um.es:fernando.perez8/gcProfileMakeR.git

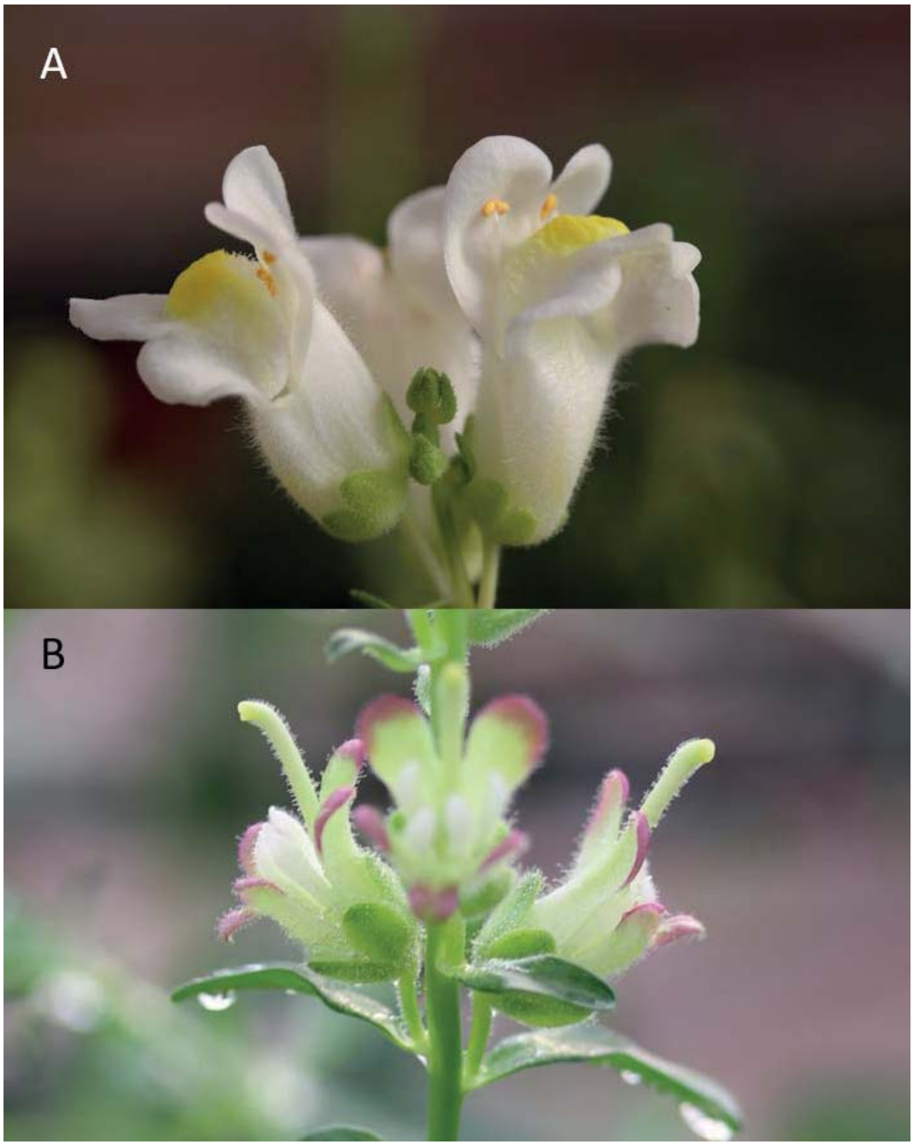

Figure 1. Flowers of *Antirrhinum*. (A) *compacta (co)*. (B) *def-nic*otianoides (*def-nic*).

Results and Discussion

The amount of work required to analyse every chromatogram by hand, and the subsequent comparison between samples makes it an error prone and a tedious process. We decided to develop a tool that would accelerate the actual identification of compounds, create the lists of constitutive and non constitutive compounds and use reproducible criteria for downstream processing and data reusability. We developed gcProfileMakeR using the R language as R is open source, and the scientific community, especially biology, is doing a massive use of it.

gcProfileMakeR uses standard formatted inputs

gcProfileMakeR uses as inputs two types of raw data: either XLS data files obtained directly from Agilent Chemstation software (Library Search Report) or CSV (separated by “;”) files with data organized as specified in Figure 2. Files corresponding to replicas should be placed together in a separated folder which must be set in the working directory. GC basic data contains information for each integrated peak about retention time (RT) and peak area. Furthermore, mass spectra (GC MS) alignment with available MS libraries allows to identify the compounds present in the sample with a certain degree of confidence (quality). Usually, annotated compounds (hits) are listed according to the quality of the match between the mass spectra obtained and the mass spectra listed in the MS library. Moreover, hits are specified by chemical names of the compound and the CAS associated to the hit/compound. CAS numbers are specific for a compound whereas chemical names are redundant and may imply different isomers or molecules. gcProfileMakeR works with RT, qualities and CAS numbers in order to provide lists of compounds identified by CAS numbers, chemical names, peak areas and qualities.

Although automatic integration should not be restricted, adequate integration of peaks is necessary to provide reliable results. Only three hits should be listed of probable compounds matching a certain peak.

Some packages are recommended to be pre installed in R before gcProfileMakeR runs: readxl, plyr, stringr, dplyr, tidyr, ggplot2 and egg.

Samples analysed can originate from any kind of tissue (i.e. genotype and/or treatment) and not necessarily GC MS metabolomics data should be analysed. Any type of technique providing results expressed by CAS numbers associated to chemical names, qualities, retention times and a measure of abundance (areas in GC MS), such as liquid chromatography, should be able to obtain reliable results.

```
Compound No.;RT;Area;Hit Name;Quality;CAS No.
9;7.654;3308324;(E)-Ocimene;98;003779-61-1
;;;(Z)-Ocimene;96;003338-55-4
;;;Ocimene;96;013877-91-3
```

Figure 2. Format for CSV inputs into gcProfileMakeR

gcProfileMaker has three functions

gcProfileMakeR uses three functions which are specified in Table 1. They need to run in the detailed order and should be assigned to different variables (i.e. p1, p2 and p3).

Table 1. gcProfileMakeR functions with default values. The order in which functions need to run is specified.

Order	Function
1st	NormalizeWithinFiles(path = getwd(), type = "xls", thr = 0.2, savefiles = FALSE, filterLowQual = 0.99)
2nd	NormalizeBetweenFiles(data, savefiles = FALSE, thr = 2, filterLowQual = 0)
3rd	getGroups(data, savefiles = FALSE, verbose = TRUE, qcutoff = 85, ncFreqCutoff = 0.3, pFreqCutoff = 0.9)
4th	plotGroup(data, compoundType = " ")

The first function NormalizeWithinFiles, analyses each file/sample and modifies it so that each file will contain for each retention time a set of possible hits (compounds). Peak areas of the same compounds found in different RT, will be added to a single value. The second function NormalizeBetweenFiles, reaches a consensus between files in such a way that same compounds separated in relatively close retention time are grouped together. This section of the program performs a pre processing of the data and reaches an agreement with the raw data obtained from each sample converting it into a set of data which can be used for grouping metabolomes into constitutive and non constitutive. Numerous programs have been released for pre processing GC MS data such as MetAlign, MZmine, SpectConnect, XCMS and GCalign and their efforts are put into the alignment, untargeted detection, matching and cataloguing of chromatographic peaks by using mainly retention times and mass spectra (Coble and Fraga, 2014; Ottensmann et al., 2017). gcProfileMakeR differs as it aligns peaks based on retention time proximities between samples, taking into account similarities of hits by CAS numbers and the quality of the matches with the MSlibrary.

The third function getGroups, establishes what is considered as “Profile”, “Non constitutive by frequency” and “Non constitutive by quality”.

Parameters can be adapted by users, according to the features of the data to be analysed. The first function, NormalizeWithinFiles (thr = 0.2, filterLowQual = 0.99) establishes the threshold (thr) of retention time to be considered in each file for each peak and the filter to be applied to secondary hits with low quality (filterLowQual) comparing to the first hit in a particular retention time. The second function, NormalizeBetweenFiles (thr = 1, filterLowQual = 0) establishes the threshold (thr) of time to be considered between all files and the filter to be applied to hits of a quality below a set value (filterLowQual), considering all the files.

The third function getGroups (qcutoff) parameter, establishes the threshold average quality that each compound has in order to be included as “Profile” or as “Non constitutive by Quality”. Parameters ncFreqCutoff and pFreqCutoff establish the ranges of frequency of compounds within the set of samples in order to group them as “Non constitutive by Frequency” and “Profile”. getGroups (ncFreqCutoff = 0.3) is used to determine the minimal frequency that establishes compounds considered as Non constitutive by Frequency”. getGroups (pFreqCutoff = 0.9) resolves which is the minimal frequency that establishes “Profile” compounds, along with getGroups (qcutoff) parameter.

Because gcProfileMakeR works with mean qualities for grouping compounds, relatively relaxed cut offs should be fixed. Special attention needs to be paid in cases where the number of samples/files is relatively low (n≈5) as percentage parameters play an important role. Exploring data before analysing is recommended.

Default values were tested with different sets of samples and number of samples and have proved the best outputs when compared to manual annotation (data not shown).

The complete workflow of gcProfileMakeR is indicated in Figure 3: inputs, functions used and outputs. The program produces three mutually exclusive lists (“Profile”, “Non constitutive by Frequency” and “Non Constitutive by Quality”) of CAS numbers shown on the console. Results can be saved on disk (getGroups(savefiles=TRUE)). By these means, four excel files are obtained: profile, nonConstitutiveQual, nonConstitutiveFreq and AuxTable. These excel files provide the list of CAS numbers along with their chemical names, except in AuxTable where CAS numbers are associated to the samples where they have appeared, along with the peak area and quality of the match.

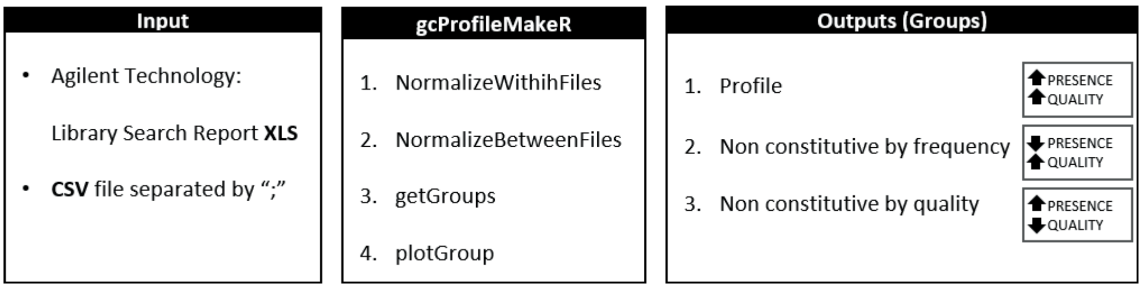

Figure 3. Workflow of gcProfileMakeR.

Compounds listed as “Profile” are those compounds which appear in a determined proportion in the samples (default: pFreqCutoff = 90%) and which have a high matching quality (default: qcutoff = 85%). Compounds listed as “Non Constitutive by Frequency” are metabolites with a relatively high quality score (default: qcutoff = 85%) in the MS analysis but present in less than 90% of the samples (default: pFreqCutoff = 90%). Finally, compounds listed as “Non Constitutive by Quality” are metabolites with a low quality (below default value: pFreqCutoff = 90%) that are in at least 30% of the samples (default: ncFreqCutoff = 30%).

Results can be plotted with the function plotGroup. In this function, compoundType parameter can be adjusted in order to get profiles (“p”), non constitutive by frequency (“ncf”) or non constitutive by quality (“ncq”). Results are plotted according to the average area and quality of each compound grouped in each category (Figures 4 and 5).

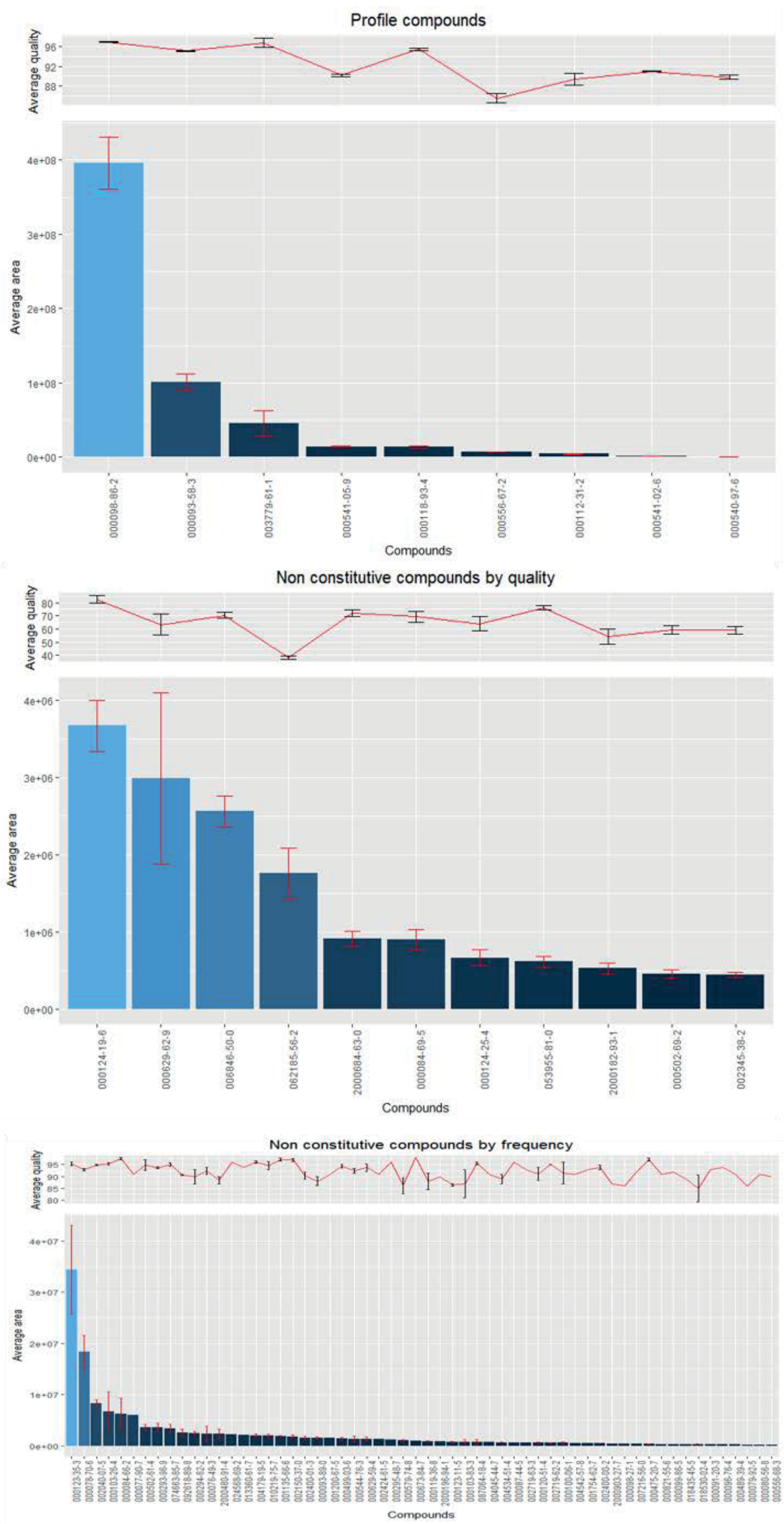

Figure 4. gcProfileMakeR results (plotResults) for *compacta* mutant flowers scent emission. Top graphics display average quality with standard deviation. Lower graphics display average area with standard deviation.

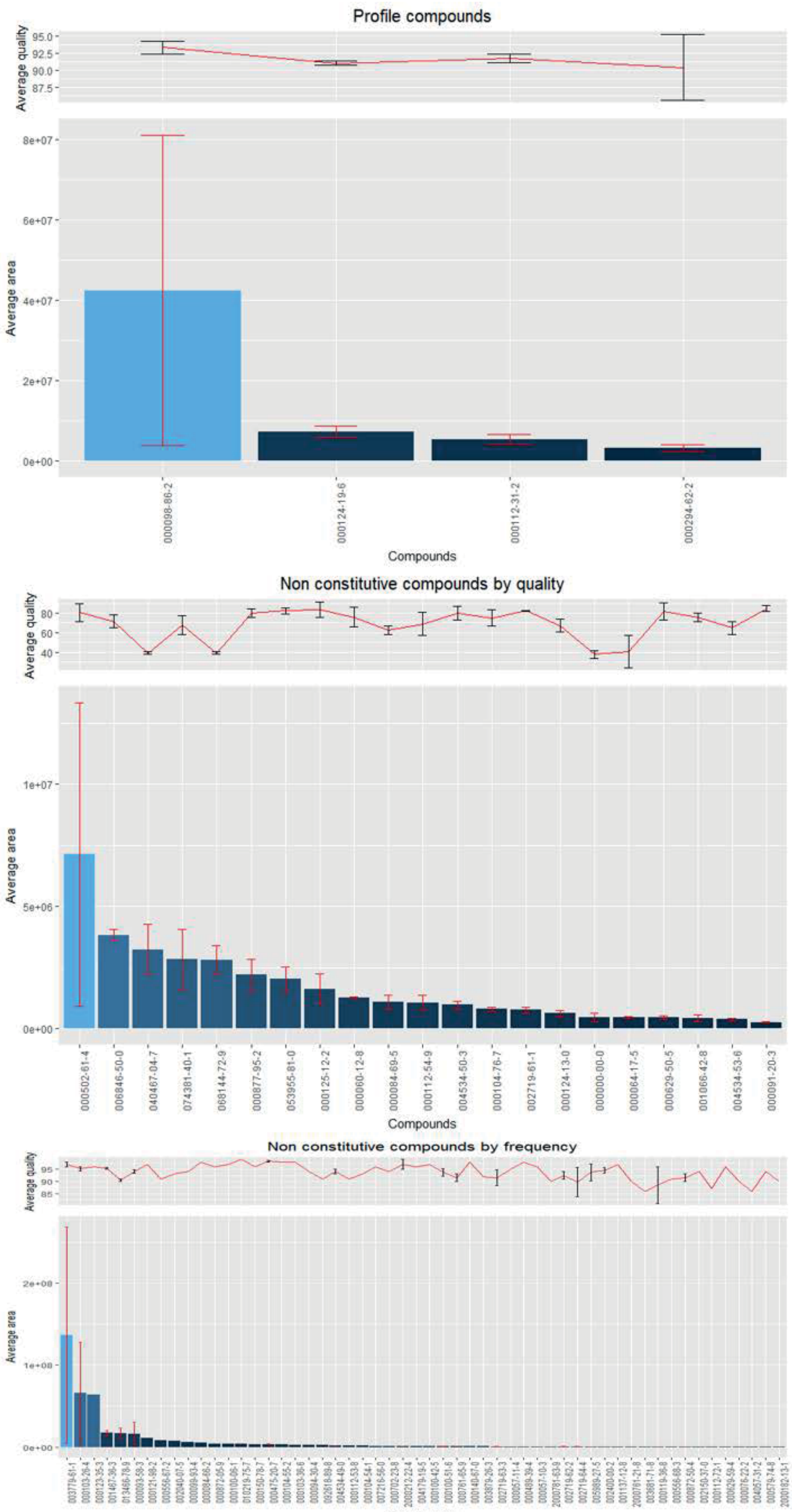

Figure 5. gcProfileMakeR results (plotResults) for *def-nicotianoides* mutant flowers scent emission. Top graphics displays average quality with standard deviation. Lower graphics display average area with standard deviation.

Compacta and *deficiens-nicotianoides* volatile metabolomes

Floral scent emission depends on properly formed petal tissues, as weak alleles of B function genes such as *deficiens nicotianoides* (Sommer et al., 1991) or *compacta* show significant changes in the quantities of the terpenoids, myrcene and ocimene, and the phenylpropanoid methyl benzoate are affected by the homeotic mutation (Manchado Rojo et al., 2012).

We analysed two datasets of floral volatiles produced by the mutants *def nic*otianoides (*def nic*) (n=9) and *compacta (co)* (n=29) differing in the quality of petal development and scent emission (Figure 1, 4 and 5). gcProfileMakeR allowed to analyse a relatively elevated number of samples much faster than manually. Manual data analysis of these CG MS samples could have lasted a few weeks. Instead by gcProfileMakeR running time for co was slightly over 36 seconds and for *def nic* was around 4 seconds on a computer with an ADM FX 8320 processor with 8 cores and 8 Gb of RAM memory.

In co mutant flowers, gcProfileMakeR originally identified 9 VOCs as “Profile”, 58 as “Non Constitutive by Quality” and 10 as “Non Constitutive by Frequency” (Figure 4 and Supplementary Table 1). We sifted through these results and removed compounds considered to not be synthesized by plants. After removing contaminant VOCs we ended up with 5 VOCs as “Profile”, 52 as “Non Constitutive by Quality” and 8 as “Non Constitutive by Frequency” (Tables 2 and 3).

On the other hand, gcProfileMakeR identified for *def nic* mutant flowers 4 compounds as “Profile”, 54 as “Non Constitutive by Quality” and 21 as “Non Constitutive by Frequency” (Figure 5 and Supplementary Table 2). After scrutiny for contaminants, figures were reduced to 48 “Non Constitutive by Quality” and 15 as “Non Constitutive by Frequency” (Tabes 2 and 4).

Table 2. gcProfileMakeR classification of VOCs as Profile and Non-Constitutive by Quality for *compacta* and *def-nicotianoides* mutants, after filtering out compounds not biosynthesized by plants.

COMPACTA		*DEF-NICOTIANOIDES*	
CAS No	Name	CAS No	Name
	Profile		
003779-61-1	(E)-Ocimene	000098-86-2	Acetophenone
000098-86-2	Acetophenone	000124-19-6	Nonanal
000093-58-3	Methyl Benzoate	000112-31-2	Decanal
000118-93-4	Acetophenone, 2'-Hydroxy	000294-62-2	Cyclododecane
000112-31-2	Decanal		
	Non-constitutive by Quality		
053955-81-0	Methyl 2-Methylbutanoate	053955-81-0	Methyl 2-Methylbutanoate
000124-19-6	Nonanal	000112-54-9	Dodecanal
000629-62-9	Pentadecane	000502-61-4	(E)-Farnesene
002345-38-2	Acetic Acid, (Trimethylsilyl)	002719-61-1	Dodecane, 2-Phenyl-
000502-69-2	Hexahydrofarnesyl Acetone	004534-50-3	Tridecane, 5-Phenyl-
000124-25-4	Tetradecanal	004534-53-6	2-Phenyltridecane
2000182-93-1	Neryl Acetone	000124-13-0	Octanal
062185-56-2	1-Hexene, 2,5,5-Trimethyl	000104-76-7	2 Ethyl Hexanol
		000091-20-3	Naphthalene
		040467-04-7	2-Hexene, 2,5,5-Trimethyl
		000125-12-2	Isobornyl Acetate
		001066-42-8	Silanediol, Dimethyl
		000060-12-8	Benzeneethanol
		000877-95-2	Acetamide, N-(2-Phenylethyl)
		000629-50-5	Tridecane

Whereas the *co* mutant scent profile was formed by (E) ocimene, acetophenone, methyl benzoate, acetophenone, 2’ hydroxy and decanal; *def nic* floral profile was composed by acetophenone, nonanal and decanal.

In addition, we were able to identify compounds that can be emitted by these genotypes but not on a regular basis. The fatty acid derived, nonanal, is emitted constitutively by *def nic*, while it is classified as non constitutive by quality in co mutants. The low average quality found in matches of nonanal from *compacta* mutants might be caused by coelution of this compound and an additional compound emitted by *co* but not by *def nic* genotypes (Lu et al., 2008). On the contrary, (E) ocimene and methyl benzoate form part of the constitutive profile of *co*, whereas in *def nic* genetic background these compounds are classified as non constitutive by frequency, as they do not trespass the threshold established as default: getGroups(pFreqCutoff =0.9).

Table 3. gcProfileMakeR classification of VOCs as Non-Constitutive by Frequency for *compacta* mutant flowers, after scrutiny of no biosynthesized compounds by plants.

	COMPACTA		
	Non-constitutive by Frequency		
000103-83-3	Benzylamine, N,N-Dimethyl	000293-96-9	Cyclodecane
000093-89-0	Benzoic Acid, Ethyl Ester	074663-85-7	Nonylcyclopropane
004179-19-5	3,5-Dimethoxytoluene	002719-63-3	5-Phenyldodecane
000499-03-6	M-Mentha-1,8-Diene	092618-89-8	Acetic Acid, Bornyl Ester
000629-59-4	Tetradecane	000087-44-5	(-)-.Beta.-Caryophyllen
000502-61-4	(E)-Farnesene	000099-86-5	Alpha.-Terpinene
018530-02-4	(-)-Campherenone	000077-90-7	Acetyl Butyl Citrate
000544-76-3	Hexadecane	000078-70-6	Linalol
002400-01-3	Benzene, (1-Hexylheptyl)	000673-84-7	Alloocimene
002719-62-2	Benzene, (1-Pentylheptyl)	000079-92-5	Camphene
000119-36-8	Methyl Salicylate	000120-51-4	Benzoic Acid, Phenylmethyl Ester
000103-26-4	Methyl Cinnamate	000821-55-6	2-Nonanone
000295-48-7	Cyclopentadecane	000123-11-5	Benzaldehyde, 4-Methoxy
001200-67-5	Isobornyl Formate	018435-45-5	1-Nonadecene
000076-49-3	Bornyl Acetate	004534-51-4	Tridecane, 4-Phenyl
000579-74-8	Acetophenone, 2'-Methoxy	002400-00-2	3-Phenyldodecane
000294-62-2	Cyclododecane	001135-66-6	Isolongifolen
013360-61-7	1-Pentadecene	000475-20-7	Longifolene
002150-37-0	Benzoic Acid, 3,5-Dimethoxy-, Methyl Ester	087064-18-4	Alloisolongifolene
000080-56-8	2-Pinene	010219-75-7	Eremophilene
000091-20-3	Naphthalene	002040-07-5	Acetophenone, 2',4',5'-Trimethyl
000100-06-1	Acetanisole	002424-61-5	2-Butenedioic Acid (Z)-, Monododecyl Ester
001754-62-7	(E)-Methyl Cinnamate	007216-56-0	Neo-Allo-Ocimene
000123-35-3	Beta.-Myrcene	004542-57-8	Dilauryl Ether
000489-39-4	Aromadendrene	000098-27-1	P-Tert-Butyl-O-Cresol
004045-44-7	1,3-Cyclopentadiene, 1,2,3,4,5-Pentamethyl-	000096-76-4	Phenol, 2,4-Di-Tert-Butyl

On the other hand, acetophenone, 2’ hydroxy has been identified as part of *co* core profile, however it has not been classified in none of *def nic* volatile metabolome groups by gcProfileMakeR indicating that it is not synthesized. Nonetheless, acetophenone, 4' hydroxy has been classified as non constitutive by quality in *def nic*.

Despite the necessity of sifting through gcProfileMakeR outputs, the direct plotting of results by means of plotGroup function allows a first glance to the samples volatile metabolome. Average abundances of each compound within each group can be observed along with CAS Registry Numbers (CAS). For instance, Figures 3 and 4 indicate that in *co* and *def nic* genotypes acetophenone (98 86 2) is the main compound, although *co* flowers, on average, emit 10 folds more acetophenone than *def nic* flowers.

Table 4. gcProfileMakeR classification of VOCs as Non-Constitutive by Frequency for *def-nicotianoides* mutant flowers, after scrutiny of no biosynthesized compounds by plants.

DEF-NICOTIANOIDES			
Non-constitutive by Frequency			
003779-61-1	(E)-Ocimene	007216-56-0	Neo-Allo-Ocimene
000093-58-3	Methyl Benzoate	000150-78-7	Benzene, 1,4-Dimethoxy
013466-78-9	3-Carene	000140-67-0	Estragole
000099-93-4	Acetophenone, 4'-hydroxy-	000100-06-1	Acetanisole
000119-36-8	Methyl Salicilate	000702-23-8	2-(4-Methoxyphenyl)Ethanol
004179-19-5	3,5-Dimethoxytoluene	000121-98-2	Benzoic Acid, 4-Methoxy-, Methyl Ester
000104-55-2	2-Propenal, 3-Phenyl	000094-30-4	Ethyl Anisate
000579-74-8	Acetophenone, 2'-Methoxy	000103-36-6	Cinnamic Acid, Ethyl Ester
000104-54-1	(Trans)-Cinnamyl Alcohol	000872-05-9	1-Decene
000103-26-4	Methyl Cinnamate	003681-71-8	3-Hexen-1-Ol, Acetate, (Z)-
003879-26-3	Cis-Geranylacetone	005989-27-5	D-Limonene
000112-53-8	Dodecanol	000100-51-6	Benzyl Alcohol
002150-37-0	Benzoic Acid, 3,5-Dimethoxy-, Methyl Ester	000761-65-9	Dibutylformamide
002719-62-2	Benzene, (1-Pentylheptyl)	001137-12-8	Longicyclene
002719-63-3	Benzene, (1-Butyloctyl)	000475-20-7	Longifolene
002719-64-4	Benzene, (1-Propylnonyl)	000489-39-4	Aromadendrene
002400-00-2	Benzene, (1-Ethyldecyl)	2000212-22-4	Trans (.Beta.)-Caryophyllene
004534-49-0	Benzene, (1-Pentyloctyl)	001467-36-3	Ethanone, 1-(2,3,4-Trimethylphenyl)
000872-50-4	2-Pyrrolidinone, 1-Methyl	002040-07-5	Ethanone, 1-(2,4,5-Trimethylphenyl)
092618-89-8	Acetic Acid, Bornyl Ester	010219-75-7	Eremophilene
000057-10-3	Palmitic Acid	000076-22-2	Camphor
000057-11-4	Octadecanoic Acid	000629-59-4	Tetradecane
000100-42-5	Cinnamene	000112-72-1	1-Tetradecanol
000123-35-3	Beta.-Myrcene	004057-31-2	Fenchyl Acetate

gcProfileMakeR gives as output a core metabolome and non-constitutive components

Previous studies determined the emission of ocimene, methyl benzoate and myrcene in *Antirrhinum co* and *def nic* mutants (Manchado Rojo et al., 2012). (E) ocimene, methyl benzoate and β myrcene are common floral volatiles (Knudsen et al., 2006) with implications on insects behaviour and inducible metabolism (Negre, 2003; Terry et al., 2007; Shimoda et al., 2012). Whereas *co* flowers emit (E) ocimene and methyl benzoate constitutively, their emission of β myrcene is classified as non constitutive by gcProfileMakeR. Moreover, *co* flowers emit constitutively acetophenone, a common floral volatile with implications in plant insect communication (The Pherobase; Erbilgin et al., 2008; Suchet et al., 2010), acetophenone, 2' hydroxy, a compound with acaricidal properties (Kim et al., 2013) and decanal, a kairomone implicated in insect attraction (Molnár et al., 2015).

Our results corroborate that *def nic* mutants are able to synthesize (E) ocimene, methyl benzoate and β myrcene, however their emission does not respond to our core metabolome category (VOCs emitted in 90% of the samples with an average matching quality with MS library above 85%). Contrastingly, core VOCs in *def nic* were acetophenone, nonanal and decanal, which seem to attract a pest of maize when acting in combination (Molnár et al., 2015) and cyclododecane which is also produced in bat pollinated plants (Galarda Varassin et al., 2001).

The differing VOC profiles would correspond to flowers that emit a scent profile somewhat similar to a wild type plant in *co* (Manchado Rojo et al., 2012), but differs strongly in *def nic* where the sepaloid identity of the second whorl organs is reflected in the major VOCs found as constitutive profile, that are usually produced by vegetative tissues.

By using gcProfileMakeR we were able to identify 5 and 4 core volatiles and a total of 60 and 63 volatiles emitted non constitutively in *co* and *def nic*, respectively. We have established the scent phenotypic space of both mutants. This allows their characterization and comparison (Weiss et al., 2016b). Non constitutive VOCs identified may be the result of inducible metabolism activated in the samples either when flowers were attached to plants or during handling for sampling of VOCs from blossoms.

gcProfileMakeR in the context of manual and automatic annotation of metabolites

Determining scent profiles of a set of samples manually is a hard task, especially if the number of samples is elevated. Manual data acquisition and analysis is, apart from slower, more error prone due to the high amount of VOCs that are emitted by plants, making it easy to drop compounds during hand curation.

Although several programs have been recently published for analysing GC MS data (Coble and Fraga, 2014; Domingo Almenara et al., 2017a; Ottensmann et al., 2017), each program has its own characteristics that may suit different users. In gcProfileMakeR, peak picking and annotation is previously done by Chemstation (or equivalent software) (Domingo Almenara et al., 2017b). gcProfileMakeR does not need an external mass spectra library as other programs do (Domingo Almenara et al., 2017a), it directly works with the matches that regular chromatographic tools provide. In fact, it is based on Agilent Chemstation XLS Library Search Report. Although it could work with any other chromatographic dataset, provided the input format is the specified in Figure 2. This new program works as a scientist working manually would work (Weiß et al., 2017). First a consensus of each sample is needed, afterwards a consensus between samples needs to be reached in order to determine which compounds form the core metabolome of the set of samples and which are not consistent enough to be listed as constitutive profile.

Furthermore, compared to current practice where most publication use chemical names, the outputs with CAS numbers should ease the implementation of the FAIR principles for data management (Wilkinson et al., 2016).

Importance of non-constitutive compounds

Compounds are listed by gcProfileMakeR as non constitutive because their presence within samples is relatively low. However their identification is both biologically and methodologically relevant. First of all, compounds which are potentially synthesised under a certain genotype (set of samples), although their presence might not be ubiquitous, are important. They may be the result of metabolic changes occurred due to numerous reasons such as insect feeding (Gaquerel et al., 2009), mechanical wounding (Mithöfer et al., 2005; Delphia et al., 2007), indirect defence activated by neighbouring plants (Kost and Heil, 2006), or even by microorganisms (Ryu et al., 2004). Consequently, their identification may lead to further understanding of changes in metabolic processes associated to different conditions.

Non constitutive results are separated into two different categories for methodological reasons. Theoretically, only compounds with a high matching quality should be considered, as it is the case of those obtained in table “Non Constitutive by Frequency”. Nevertheless, annotation of compounds has not been fully developed yet and there might be matchings with low qualities caused by convolutions of similar compounds or incomplete information stored in the mass spectra (MS) libraries. This is the case of methyl jasmonate in mass spectral databases Willey10th NIST11b (Agilent Technologies, Wilmington, USA). Despite being an important metabolite implicated in plant defense (Degenhardt and Lincoln, 2006), mass spectra of methyl jasmonate in the mentioned MS library is incomplete because just main masses are stored. Therefore, even GC MS analysis of the methyl jasmonate standard (Sigma Aldrich, 39924 52 2) obtains a library matching quality of 53% under a gas chromatographic working environment (Figure 6). Furthermore, more updated MS libraries may have better scores for the current chromatograms, allowing reanalysis of the data corresponding to Non Constitutive by Quality.

Additionally, some methodological issues may affect to the acquisition of volatile compounds, as it is the case of VOCs with (high/low) polarity and their adsorption by polidmethylsiloxane stirbars (Sgorbini et al., 2012). For all those reasons, gcProfileMakeR lists compounds which appear frequently in the set of samples but with low quality, these compounds are accounted as “Non Constitutive by Quality”.

A

B

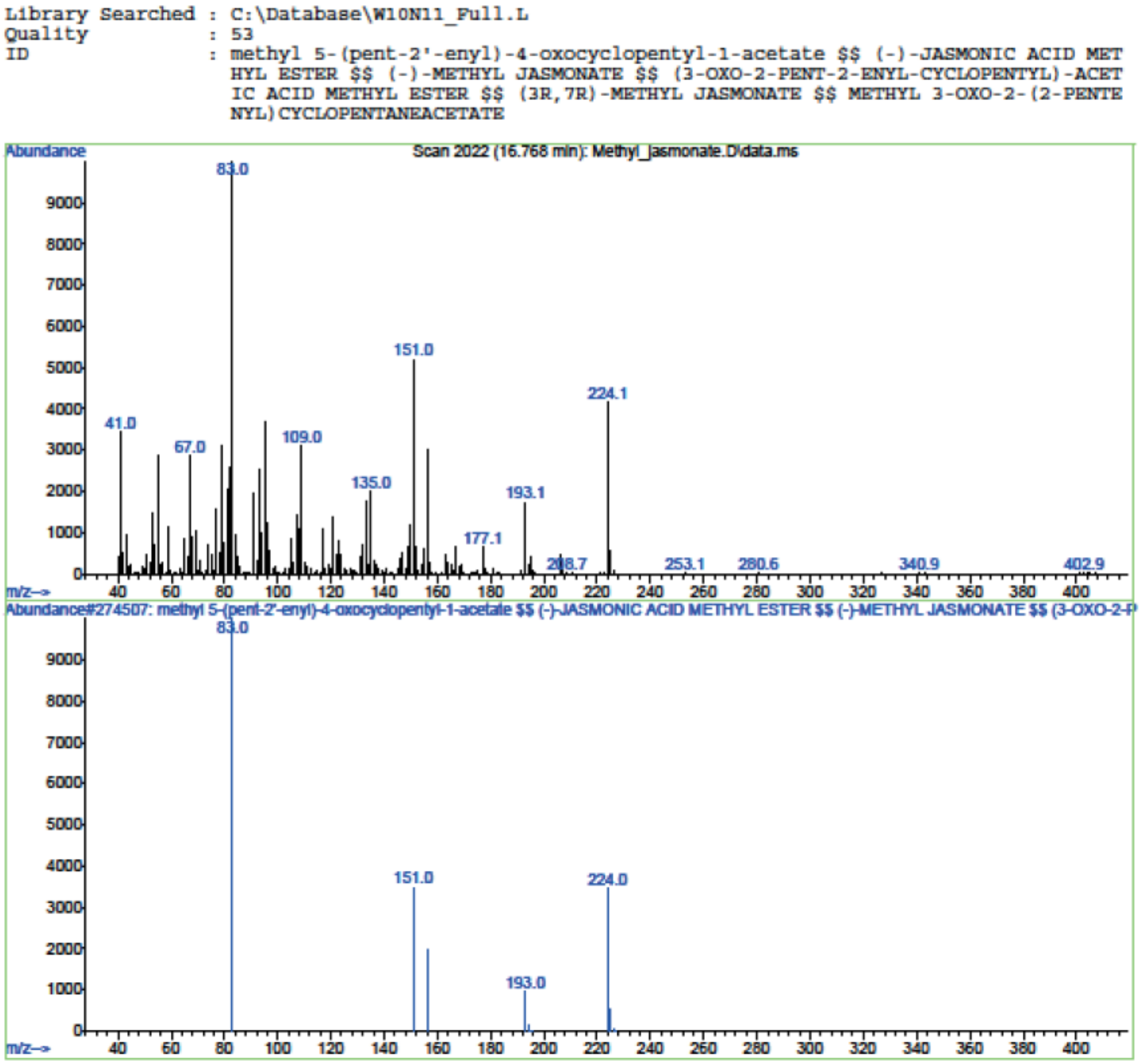

Figure 6. Mass spectra of methyl jasmonate (CAS No: 39924-52-2). (A) commercial standard, (B) mass spectral databases Willey10th-NIST11b (Agilent Technologies, Wilmington, USA).

Pitfalls

gcProfileMakeR has been conceived to work with screenings or untargeted metabolomes, hence is a tool that provides information about compounds which probably appear in a set of samples with a certain quality of matching between mass spectra and libraries (Barba et al., 2017). Nevertheless, further injection of standards should be done in order to ensure peak retention time and perform adequate quantifications. However, complex matrices as plants can behold metabolomes so diverse that each compound emitted is not feasible to be allocated to a pure standard (Roessner et al., 2000; Raffo et al., 2018; Ruiz Hernández et al., 2018)

In spite of obtaining definitive output data with gcProfileMakeR, researchers need to process them. Especially in cases where MS libraries are incomplete or convolutions of compounds occur. Furthermore, some contaminating compounds might be listed and need to be identified and eliminated for final results. These compounds can be the result of column bleed as well as contaminants present during samplings (Hardesty et al., 2015).

Conclusions

We were able to characterize two common *Antirrhinum* mutants by their core and non constitutive volatile metabolomes, establishing their scent phenotypic space. gcProfileMakeR can help in establishing clear genotype phenotype metabolic profiles for genetic and environmental experiments. gcProfileMakeR gives a first approximation to biologically relevant metabolomics data by using common formats (.xlsx/.csv) and implementing the FAIR Data Principles.

List of author contributions

V.R H, S.A G and M.E C. conceived the original screening and research plans; V.R H. supervised the experiments; F.P S. coded the application with help from P.J.N; V.R H and S.A G. V R H designed the experiments and analysed the data; V.R H. J.W. and M.E C. wrote the manuscript. All authors corrected the manuscript. JW, MEC and PJN wrote the grant applications. M.E C. agrees to serve as the author responsible for contact and ensures communication.

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Supplementary material

Supplementary Table S1. *Compacta* mutant flowers full results from gcProfileMakeR.

COMPACTA	
Profile	
000541-05-9	Cyclotrisiloxane, hexamethyl
000556-67-2	Cyclotetrasiloxane, octamethyl
003779-61-1	(E)-Ocimene
000098-86-2	Acetophenone
000093-58-3	Methyl benzoate
000541-02-6	Cyclopentasiloxane, decamethyl
000118-93-4	Acetophenone, 2'-hydroxy
000112-31-2	Decanal
000540-97-6	Cyclohexasiloxane, dodecamethyl
Non-constitutive by Frequency	
000103-83-3	Benzylamine, N,N-Dimethyl
000093-89-0	Benzoic Acid, Ethyl Ester
004179-19-5	3,5-Dimethoxytoluene
000499-03-6	M-Mentha-1,8-Diene
000629-59-4	Tetradecane
000502-61-4	(E)-Farnesene
018530-02-4	(-)-Campherenone
000544-76-3	Hexadecane
2000903-27-7	1-Tetratriacontanol, Pentafluoropropionate
002400-01-3	Benzene, (1-Hexylheptyl)
002719-62-2	Benzene, (1-Pentylheptyl)
000119-36-8	Methyl Salicylate
000103-26-4	Methyl Cinnamate
000295-48-7	Cyclopentadecane
001200-67-5	Isobornyl Formate
000076-49-3	Bornyl Acetate
000579-74-8	Acetophenone, 2'-Methoxy
000294-62-2	Cyclododecane
013360-61-7	1-Pentadecene
2000480-91-4	(14beta)-Pregnane
002150-37-0	Benzoic Acid, 3,5-Dimethoxy-, Methyl Ester
2000196-94-1	1-Allyl-3-Methylindole-2-Carbaldehyde
000080-56-8	2-Pinene
000091-20-3	Naphthalene
000100-06-1	Acetanisole
001754-62-7	(E)-Methyl Cinnamate
000123-35-3	Beta.-Myrcene
000489-39-4	Aromadendrene
004045-44-7	1,3-Cyclopentadiene, 1,2,3,4,5-Pentamethyl-
000293-96-9	Cyclodecane

074663-85-7	Nonylcyclopropane
002719-63-3	5-Phenyldodecane
092618-89-8	Acetic Acid, 1,7,7-Trimethyl-Bicyclo[2.2.1]Hept-2-Yl Ester
000087-44-5	(-)-.Beta.-Caryophyllen
000099-86-5	Alpha.-Terpinene
024568-69-2	(+-)-Seychellene
000077-90-7	Acetyl Butyl Citrate
000078-70-6	Linalol
000673-84-7	Alloocimene
000079-92-5	Camphene
000120-51-4	Benzoic Acid, Phenylmethyl Ester
000821-55-6	2-Nonanone
000123-11-5	Benzaldehyde, 4-Methoxy
018435-45-5	1-Nonadecene
004534-51-4	Tridecane, 4-Phenyl
002400-00-2	3-Phenyldodecane
001135-66-6	Isolongifolen
000475-20-7	Longifolene
087064-18-4	1,4-Methano-1H-Indene, Octahydro-1,7a-Dimethyl-4-(1-Methylethenyl)-, [1S-(1.Alpha.,3a.Beta.,4.Alpha.,7a.Beta.)]-
010219-75-7	Eremophilene
002040-07-5	Acetophenone, 2',4',5'-Trimethyl
000556-68-3	Cyclooctasiloxane, Hexadecamethyl
002424-61-5	2-Butenedioic Acid (Z)-, Monododecyl Ester
007216-56-0	Neo-Allo-Ocimene
000084-66-2	Diethyl Phthalate
004542-57-8	Dilauryl Ether
000098-27-1	P-Tert-Butyl-O-Cresol
000096-76-4	Phenol, 2,4-Di-Tert-Butyl
Non-constitutive by Quality	
053955-81-0	Methyl 2-Methylbutanoate
000629-62-9	Pentadecane
000084-69-5	Diisobutyl Phthalate
002345-38-2	Acetic Acid, (Trimethylsilyl)
000502-69-2	Hexahydrofarnesyl Acetone
000124-25-4	Tetradecanal
2000684-63-0	Phthalic Acid, Decyl Isobutyl Ester
006846-50-0	Txib
2000182-93-1	Neryl Acetone
062185-56-2	1-Hexene, 2,5,5-Trimethyl

Supplementary Table S2. *Def-nicotianoides* mutant flowers full results from gcProfileMakeR.

DEF-NICOTIANOIDES	
Profile	
000098-86-2	Acetophenone
000124-19-6	Nonanal
000112-31-2	Decanal
000294-62-2	Cyclododecane
Non-constitutive by Frequency	
003779-61-1	(E)-Ocimene
000093-58-3	Methyl Benzoate
013466-78-9	3-Carene
000099-93-4	4-Hydroxy-Acetophenone
000119-36-8	Methyl Salicilate
004179-19-5	3,5-Dimethoxytoluene
000104-55-2	2-Propenal, 3-Phenyl
000579-74-8	Acetophenone, 2'-Methoxy
000104-54-1	(Trans)-Cinnamyl Alcohol
000103-26-4	Methyl Cinnamate
003879-26-3	Cis-Geranylacetone
000112-53-8	Dodecanol
002150-37-0	Benzoic Acid, 3,5-Dimethoxy-, Methyl Ester
002719-62-2	Benzene, (1-Pentylheptyl)
002719-63-3	Benzene, (1-Butyloctyl)
002719-64-4	Benzene, (1-Propylnonyl)
002400-00-2	Benzene, (1-Ethyldecyl)
004534-49-0	Benzene, (1-Pentyloctyl)
000872-50-4	2-Pyrrolidinone, 1-Methyl
092618-89-8	Acetic Acid, 1,7,7-Trimethyl-Bicyclo[2.2.1]Hept-2-Yl Ester
000057-10-3	Palmitic Acid
000057-11-4	Octadecanoic Acid
000100-42-5	Cinnamene
000123-35-3	Beta.-Myrcene
007216-56-0	Neo-Allo-Ocimene
000150-78-7	Benzene, 1,4-Dimethoxy
000140-67-0	Estragole
2000781-63-9	2-(.Beta.-D-Galactopyranosylthio)-5,6,7,8-Tetrahydrobenzothieno[2,3-D]Pyrimidine-4-Thione
000100-06-1	Acetanisole
000702-23-8	2-(4-Methoxyphenyl)Ethanol
000121-98-2	Benzoic Acid, 4-Methoxy-, Methyl Ester
2000761-21-8	2,4-Bis(4-Chlorophenyl)-5,6-Dihydrobenzo[H]Quinazoline
000094-30-4	Ethyl Anisate
000103-36-6	Cinnamic Acid, Ethyl Ester
000872-05-9	1-Decene
000556-68-3	Cyclooctasiloxane, Hexadecamethyl

003681-71-8	3-Hexen-1-Ol, Acetate, (Z)-
005989-27-5	D-Limonene
000100-51-6	Benzyl Alcohol
000761-65-9	Dibutylformamide
001137-12-8	Longicyclene
000475-20-7	Longifolene
000489-39-4	Aromadendrene
2000212-22-4	Trans (.Beta.)-Caryophyllene
001467-36-3	Ethanone, 1-(2,3,4-Trimethylphenyl)
002040-07-5	Ethanone, 1-(2,4,5-Trimethylphenyl)
010219-75-7	Eremophilene
000084-66-2	Diethyl Phthalate
2000182-13-1	3-Tert-Butyl-2-Methoxy-5-Methylphenol
000076-22-2	Camphor
000629-59-4	Tetradecane
000112-72-1	1-Tetradecanol
000556-67-2	Octamethylcyclotetrasiloxane
004057-31-2	Fenchyl Acetate
Non-constitutive by Quality	
000064-17-5	Ethanol
000112-54-9	Dodecanal
000502-61-4	(E)-Farnesene
074381-40-1	Propanoic Acid, 2-Methyl-, 1-(1,1-Dimethylethyl)-2-Methyl-1,3-Propanediyl Ester
002719-61-1	Dodecane, 2-Phenyl-
004534-50-3	Tridecane, 5-Phenyl-
000084-69-5	Diisobutyl Phthalate
004534-53-6	2-Phenyltridecane
000124-13-0	Octanal
000104-76-7	2 Ethyl Hexanol
000091-20-3	Naphthalene
006846-50-0	Txib
040467-04-7	2-Hexene, 2,5,5-Trimethyl
000125-12-2	Isobornyl Acetate
001066-42-8	Silanediol, Dimethyl
000060-12-8	Benzeneethanol
000877-95-2	Acetamide, N-(2-Phenylethyl)
000629-50-5	Tridecane
068144-72-9	2-Ethylhexyl Dichloroacetate
000000-00-0	Korytan

Chapter V

Behavioural Responses of Pest and Pollinator to Antirrhinum Floral Scent Profiles

Behavioural responses of pest and pollinator to *Antirrhinum* floral scent profiles

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Abstract

Plant volatiles play a dual role in deterring pests and attracting pollinators. Due to the importance that floral traits have on insect visits to sustain ecosystems and yields, we analysed volatile organic compounds (VOCs) mediating attraction and repellence of bumblebees (*Bombus terrestris audax*) and thrips (*Frankliniella occidentalis*). We followed a holistic approach and assessed the effect on insect preferences of different floral scent profiles from *Antirrhinum* plants. We selected the species *A. majus, A. linkianum* and four recombinant inbred lines (RILs) differing in the emission of ocimene, methyl benzoate, acetophenone and methyl cinnamate. Detailed analysis revealed the segregation of 22 VOCs comprising core scent profiles in the studied lines. Results indicate that whilst bumblebees were attracted by methyl benzoate, thrips were repelled by a higher emission of β myrcene. Furthermore, we identified compounds within blends that may act as attractants or deterrents on bumblebees and thrips. Bumblebees might be attracted to scent blends containing o acetanisole, decanal, ethyl benzoate and o acetylphenol. Other combinations including eremophylene, α farnesene, methyl cinnamate and sabinene may act as bee repellents. Moreover, scent blends which seemed to attract thrips included cinnamyl alcohol, α farnesene and methyl cinnamate, while o acetanisole may act in combination as a repellent of thrips. Our results are a first step towards breeding programs aimed at pest and pollination managements.

Introduction

Studies indicate that thirty five per cent of global crop yields rely on animal pollination and there are evidences of the positive effect of animal pollination over yield quantities and qualities (Klein et al., 2007; Klatt et al., 2013). In some places, like UK and USA, animal pollination of crops is carried out mainly by wild pollinators (Winfree et al., 2007; Breeze et al., 2011). Current work shows that wild pollinator populations have been declining over the last few years due to multiple pressures such as lack of food resources, diseases and pesticides (Vanbergen et al., 2013; Ollerton et al., 2014). Bees are important pollinators in both natural environments and for crop production. Among the relevant bee pollinators are the well known *Bombus* spp., which are broadly used in agronomic productions like tomato, *Cucurbita* spp., cocoa or vanilla (Garibaldi et al., 2011; Morse et al., 2012).

Studies on important floral characters for pollinators attraction can help breeders to select important traits that may boost crop pollination, yield and fruit quality (Bailes et al., 2015; Bailes et al., 2018). Scent is a relevant phenotypic trait in flowers as plants use aromas to mediate the attraction of mutualists and the repellence of antagonists. Levels of emission seem to be important for insect pollination, although the composition of scent profiles seems to be more relevant than the quantities (Majetic et al., 2009). Additional studies indicate that more fragrant flowers of *Cucurbita pepo* are more attractive to florivores (Theis and Adler, 2012). Therefore studies on floral scents should pay attention to both relative quantities of emission and composition of scent profiles.

Among flower feeders, the western flower thrips, *Frankliniella occidentalis*, is considered a global spread pest. This insect has a wide plant host range and their damages are produced by feeding, oviposition and transmission of diseases (Morse and Hoddle, 2006). However, they have also been described as pollinators of the point leaf manzanita, *Arctostaphylos pungens* (Eliyahu et al., 2015). Much research has been done on pesticides against thrips (Espinosa et al., 2002; Puinean et al., 2013; Guillén et al., 2014), including some common Volatile Organic Compounds (VOCs) such as p cymene, which is toxic to thrips (Janmaat et al., 2002).

Both pollinators and herbivores exert pressures in the selection of floral traits. In both cases, their behaviour may affect the maintenance of individual traits, such as an individual VOC, or mixtures of traits, such as scent blends (Farré Armengol et al., 2013). The complexity of signals produced by flowers mediates with floral visitors and for investigating these relationships, holistic approaches are needed (Junker and Parachnowitsch, 2015). The development of statistical tools permit the analysis of multiple factors at a time and are specially required for the identification of mixtures of VOCs which can be under pressure for selection (Junker et al., 2016).

In the present study, we used two species of *Antirrhinum*, *A. majus* and *A. linkianum* and four recombinant inbred lines (RILs) from a cross between these two species (Ruiz Hernández et al., 2017). We identified constitutively emitted VOCs from each line and selected the lines based on differing production of ocimene, methyl benzoate, acetophenone and methyl cinnamate. The aim of this work was to pinpoint VOCs involved in the attraction and/or repellence of bumblebees and thrips.

Materials and methods

Recombinant Inbred Lines

A cross between *A. majus* and *A. linkianum* was performed (Ruiz Hernández et al., 2017). Four selected recombinant inbred lines (RILs) on F5 F7 populations were used for further experiments: 9, 80, 112 and 113 (Figure 1).

Experimental design

Experiments were carried out in pairwise comparisons according to Figure 2. Flowers used for GC MS analysis and experiments were collected at their maximum stage of scent production, 3 4 days after anthesis (Weiss et al., 2016).

Figure 1. *Antirrhinum* flowers from *A. majus*, *A. linkianum*, RIL 112, RIL 113, RIL 9 and RIL 80.

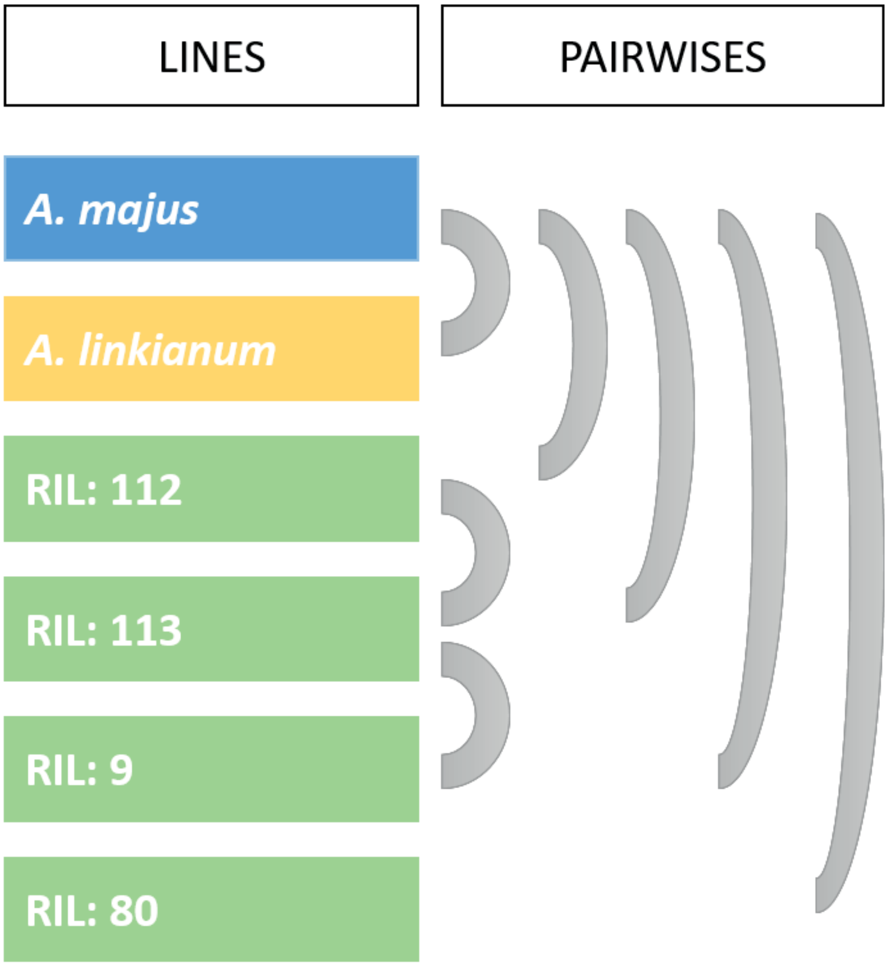

Figure 2. Experimental design in pairwise comparisons indicated by grey bars.

VOC analysis

VOCs were analysed by GC MS in 24 hours sampling periods as described in Ruiz Hernández 2017 and 2018. Flowers were placed in 2 litre desiccators located inside a growth chamber under a regime of 16:8 light dark and 23–18ºC conditions. A minimum of 10 replicas (5 from F5 and 5 from F7) from different plants within each line were analysed by GC MS.

Scent profiles were determined using the R package gcProfileMakeR (Chapter IV). Compounds present in at least 90% of the replicas, with a minimum average quality of 90%, were selected as representatives of the scent profile of each line. Contaminants were removed from the dataset. Linear retention indexes (LRI) were calculated for identifying compounds by comparing with retention times (RT) of C8 C20 alkanes (Sigma

Aldrich, 04070) (Zellner et al., 2008; Ruiz Hernández et al., 2018). *N* alkanes were analysed under the same chromatographic conditions as flower samples (Table 1).

Table 1. Profile compounds found in *A. majus*, *A. linkianum* and RILs 112, 113, 80 and 9. CAS numbers, retention times (RT), linear retention indexes (LRI) calculated and compared with bibliography

Compound	CAS No	RT	LRI calculated	LRI bibliography	Reference
Methyl 2-methyl butyrate	868-57-5	2.101	779	774	(Triskelion, 2018)
Sabinene	3387-41-5	5.034	961	976	(Acree and Arn)
β-myrcene	123-35-3	5.473	982	991	(Adams, 1995)
(E)-ocimene	3779-61-1	6.773	1042	1038	(Acree and Arn)
Acetophenone	98-86-2	7.139	1060	1065	(Adams, 1995)
Methyl benzoate	93-58-3	7.763	1089	1091	(Adams, 1995)
Linalool	78-70-6	7.844	1093	1098	(Adams, 1995)
Nonanal	124-19-6	7.954	1098	1104	(Su Rae Lee, Carlos Macku, 1991)
o-acetylphenol	118-93-4	9.039	1156	1160	(Triskelion, 2018)
Ethyl benzoate	93-89-0	9.196	1165	1185	(Acree and Arn)
Methyl salicylate	119-36-8	9.597	1187	1190	(Adams, 1995)
Decanal	112-31-2	9.792	1197	1204	(Heinen, 1990)
Benzenepropanol	122-97-4	10.235	1224	1221	(Kim et al., 2016)
3,5-dimethoxytoluene	4179-19-5	10.815	1260	1260	(Kim et al., 2016)
(E)-cinnamaldehyde	14371-10-9	10.887	1264	1283	(Acree and Arn)
Methyl hydrocinnamate	103-25-3	10.959	1269	1270	(Lima Neto et al., 2017)
o-acetanisole	579-74-8	11.235	1286	1285	(Kim et al., 2016)
Cinnamyl alcohol	104-54-1	11.320	1291	1312	(Acree and Arn)
Methyl cinnamate	103-26-4	12.525	1372	1379	(Acree and Arn)
Eremophilene	10219-75-7	14.068	1483	1490	(Acree and Arn)
α-farnesene	502-61-4	14.273	1499	1500	(Acree and Arn)
Hexahydrofarnesyl acetone	502-69-2	18.254	1835	1770	(Acree and Arn)

Bumblebee experiments

Pretraining of bees

Bombus terrestris audax colonies were obtained from Biobest Group NV (Westerlo, Belgium) and connected by a transparent tube to the flight arena, a 0.3 × 0.75 × 1.12 m plywood with a clear UV transparent Plexiglass lid (Bailes et al., 2015). Colonies were fed *ad libitum* with ~30% w/w sugar solution and pollen. Before the experiments, bees were trained in feeding from 13 cm tall feeding towers composed of black card wrapped with black tape sitting within 'Aracon' bases (Lehle, Roundrock, TX). Towers were covered with plastic mesh supporting a microcentrifuge tube lid (Figure 3) containing sucrose solution. Tower height was defined as bumblebees cannot successfully determine visual cues beyond 10 cm (Chittka and Raine, 2006). Scent experiments with bees were carried out by hiding flowers inside the towers (1:1). Experimentalist differentiated towers from each other because half of them were marked with vertical black tape stripes. Tower feeding female forager bees were marked on the thorax with water soluble paints and used for further experiments. Some bees were used more than once and, in those cases, at least 7 days with no experimenting were left between bee assays.

Figure 3. Towers used to hide flowers and perform scent-related experiments with bumblebees.

Preference assessments

Preferences of bumblebees were assessed in pairwise comparisons according to Figure 2. In the arena, 5 towers with flowers of one line and 5 towers with flowers of a different one were distributed pseudo randomly. Just one bee at a time performed the experiment. For each pairwise, at least 10 bees repeated the experiment. Microcentrifuge tube lids were supplied with 20 µl of sucrose solution. Each bee was allowed to feed 10 times and choices were recorded. Each time a bee fed from a tower, sugar solution was refilled and distribution of towers was changed. In order to control a possible preference for a specific black tape design of towers, flowers were changed in each tower pairwise. Thus, half of the bees were assigned for instance to *A. majus* non striped towers, and *A. linkianum* striped towers and the other half did the experiments the other way around. Between pairwise comparisons and changes of flowers contained in the towers, towers were cleaned with a 40% ethanol solution and left to dry to remove scent marks. Flowers inside towers were kept in contact with cotton dampened in a 5 % sucrose solution to keep them fresh.

We assessed the preference of bees for towers with and without flowers. For this experiment, different types of flowers were used in each replica. In this case, towers with no flowers were totally new. The sugary dampened cotton was added to towers without flowers as well, to control for possible preferences produced by it.

Differential conditioning

To assess the ability of bumblebees to distinguish between scents of some of the lines, we performed two differential conditioning experiments were bumblebees had to associate a certain scent with a reward or a punishment. This way, if they can differentiate between floral scents, they will learn which odour is associated to a reward and which is associated to a punishment. Experiments were performed in pairs using lines *A. majus* vs. RIL 9 and RIL 112 vs. RIL 113. In the arena, 5 towers with flowers of one line and 5 towers with flowers of the other were distributed pseudo randomly. Just one bee at a time performed the experiment. For each pairwise, 10 bees repeated the experiment. Microcentrifuge tube lids were supplied either with 20 µl of 30% sucrose solution, as reward, or with 20 µl quinine hemisulfate solution (0.12%), as punishment. Bumblebees made between 41 100 choices (with the majority of them choosing more than 80 times). Each time a bee fed from a tower, sugar solution was refilled and distribution of towers was changed. To control for towers black tape design preferences, hidden lines of flowers were changed in each pairwise.

To control for bumblebees ability to discriminate from sucrose and quinine solutions before landing and towers black tape design, we performed the same differential conditioning experiments with flowers of the same line. Thus, flowers with the same scent were hidden in sucrose and quinine supplied towers and bumblebees had to make choices. If bumblebees were not able to differentiate quinine or sugar supplied towers, then they would make choices irrespectively to towers supplemented with quinine or sugar (50 50%). The control experiment was done by 5 independent bees and they made 98 100 choices.

Thrips experiments

Field populations of thrips (*Frankliniella occidentalis*) collected in Murcia were reared for two generations in the lab (Espinosa et al., 2002). Thrips used were females and flower naïve. Experiments were performed in pairwise comparisons (Figure 2). We used new 0.5 litre plastic boxes with 1 flower (without stamen) from each line, as illustrated in Figure 4, 30 thrips in each pairwise analysis (n: 4 9) were added. Thrips were kept inside plastic boxes for 24 hours. After that, thrips were kept in the freezer for a maximum of 5 minutes (to stop their movements without killing them) and counted the number of them in each flower and in the immediate surroundings.

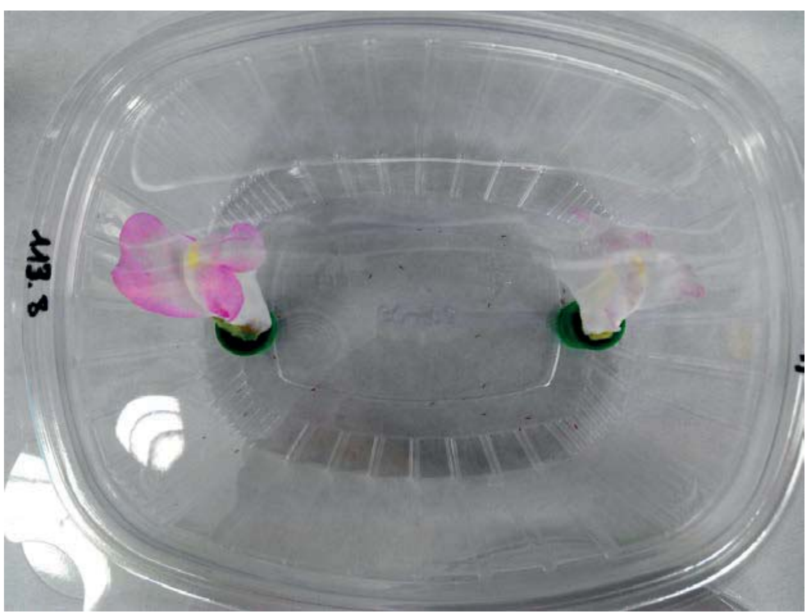

Figure 4. Experimental set up used for preferences assessments with thrips.

Additional experiments (n = 3) were carried out by adding commercial standards to low emitting acetophenone and methyl cinnamate lines (Sigma Aldrich: 96410 and 42163, respectively), comparing to the rest of the lines analysed. Standards were diluted in methanol and 80 µg of standards were supplemented inside the flowers. Quantities added were calculated according to estimations based on high emitting flowers. We provided RIL 9 and 112 flowers with acetophenone to test its effect on thrips preferences. In both cases, a flower was supplemented with the standard and the other flower (from the same line) inside the plastic box was not supplemented. Equal experiments were carried out with flowers from RIL 112 and 80 supplemented with methyl cinnamate.

Statistical analysis

Statistical analysis were performed in R (R Development Core Team, 2011) using version 3.3.3. Total scent and methyl benzoate emission were tested with ANOVA plus Tukey HSD. Average emission of β myrcene was assessed by Welch Anova due to heteroscedasticity and post hoc analysis was performed by t test.

Independent VOCs differences between lines were analysed with the dynRB package with default values (Junker et al., 2016). Due to the effect of individual VOCs on insect attraction/repellence, correlating compounds were not removed from the dataset (Chen et al., 2009). Due to lack of compliance of assumptions in the distribution of data such as normality, no PCA within the dynRB package was performed. To clarify, we show data just from port(A, B).

Bumblebee pairwise preferences were analysed using Wilcoxon test (Strauch et al., 2014).

In differential conditioning experiments, we tested if the probability of success varies with choice number for both the learning test and control experiments. We fitted a generalised linear mixed model with a binomial error structure with success or failure as response, choice number as predictor and bee identity as a random effect. Models were fit using the lme4 package (Bates et al., 2014). Differing numbers of choices in learning experiments (between 41 and 100) created difficulties with the lme4 model fitting process. We therefore fitted the model to the first 50 choices in learning experiments and used 100 choices in the control experiment.

Thrips preferences were assessed by chi square analysis (Pérez Hedo et al., 2015a; Pérez Hedo et al., 2015b).

Results

Scent profiles and total emission

There are two inbred lines from *A. majus* extensively used in *Antirrhinum* laboratory research, the Sippe 50 and 165E (Schwarz Sommer et al., 2003). The 165E line was used to create a recombinant inbred line with the wild species *A. linkianum*. The scent analysis of wild type accessions and RILs was based on several criteria including total scent emission, scent mixtures and single VOCs differences between lines. The most scented lines were RIL 9 and RIL 80, while the lowest emitters were *A. majus*, *A. linkianum*, RIL 112 and RIL 113 (Figure 5).

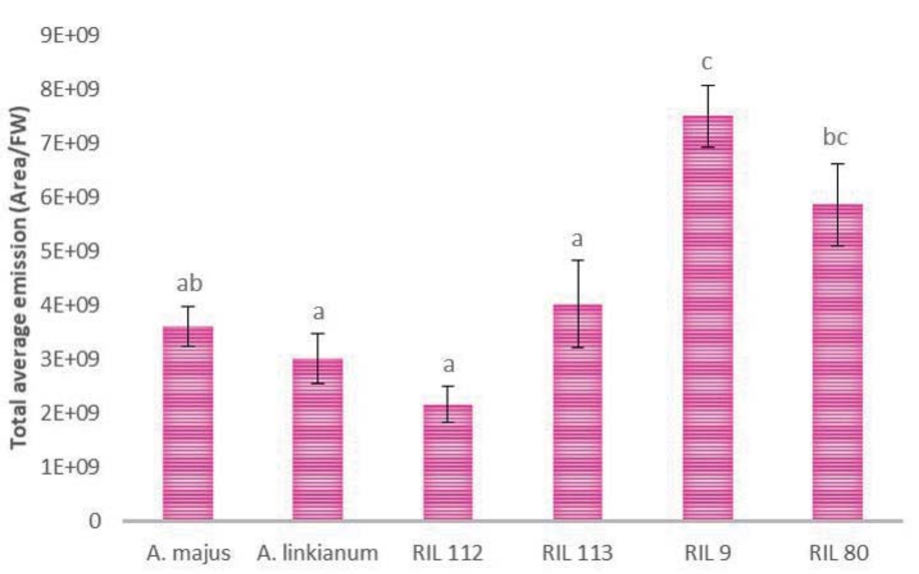

Figure 5. Total average emission of scent profiles from *A. majus*, *A. linkianum*, RIL 112, RIL 113, RIL 9 and RIL 80. Letters indicate statistical significantly different results, according to Tukey HSD. Results are expressed as integrated areas by fresh weight (FW).

Parental lines, *A. majus* and *A. linkianum* differ in the emission of ocimene, methyl benzoate, acetophenone and methyl cinnamate (Ruiz Hernández et al., 2017). Additional VOCs emitted constitutively in the parental lines were segregating in RILs, as o acetylphenol or eremophilene. Some of the VOCs found were shared by all lines, as it is the case of (E) ocimene or β myrcene. Furthermore, some VOCs not emitted constitutively in parental lines were found in RILs, such as methyl salicylate which is emitted in RILs 113, 80 and 9, benzenepropanol which is emitted constitutively in RILs 112 and 9 or 3,5 dimethoxytoluene and linalool which are emitted exclusively in RILs 80 and 9, respectively (Figure 6, Supplementary table 1). A total of 22 different VOCs were detected as part of the scent profiles of the different lines analysed (Table 1). Parental lines produced constitutively 12 out of the total amount of 22 VOCs identified. Core scent profiles of different lines varied in the number of VOCs emitted (Figure 6). Floral scent profiles of parental lines were less complex than those of their descendants. Scent profiles were composed of 9 VOCs in *A. majus*, 7 in *A. linkianum*, 12 in RIL 112, 14 in RIL 113, 16 in RIL 9 and 13 in RIL 80.

Main VOCs can be considered as those that compose around an 80 % of the total quantity emitted within a scent profile. They comprise the major and essential footprints of each of the lines analysed. Contrastingly, minor VOCs contribute in a subtle manner to scent profiles and can be classified as those whose sum contribute with around 20 % of the total amount of emission. Despite contributing in lower amounts to scent profiles they might have an important effect on flower insect interactions and are considered in the analysis. Results indicated that in parental line *A. majus* main VOCs were (E) ocimene, methyl benzoate and acetophenone, whereas in *A. linkianum* were (E) ocimene and α farnesene. Whilst minor VOCs in parental *A. majus* were composed of β myrcene, α farnesene, ethyl benzoate, o acetylphenol, o acetanisole and decanal; minor VOCs in parental *A. linkianum* were methyl cinnamate, β myrcene, eremophilene, acetophenone and sabinene (Figure 6, Supplementary Table 1).

The classification of the scent profiles of RILs (112, 113, 9 and 80) into main and minor VOCs, in general, indicates that these lines produced as major VOCs those which were emitted constitutively in either *A. majus, A. linkianum* or in both lines. However, cinnamyl alcohol was exclusively found in RILs. Main VOCs emitted by RIL 112 were (E) ocimene, methyl benzoate, α farnesene, acetophenone and methyl cinnamate, whereas in RIL 113 were acetophenone, α farnesene, (E) ocimene and methyl benzoate. Contrastingly, most abundant compounds in RIL 9 were (E) ocimene, cinnamyl alcohol, α farnesene, eremophilene and methyl benzoate, whereas in RIL 80 were acetophenone, α farnesene, methyl benzoate and cinnamyl alcohol (Figure 6, Supplementary Table 1).

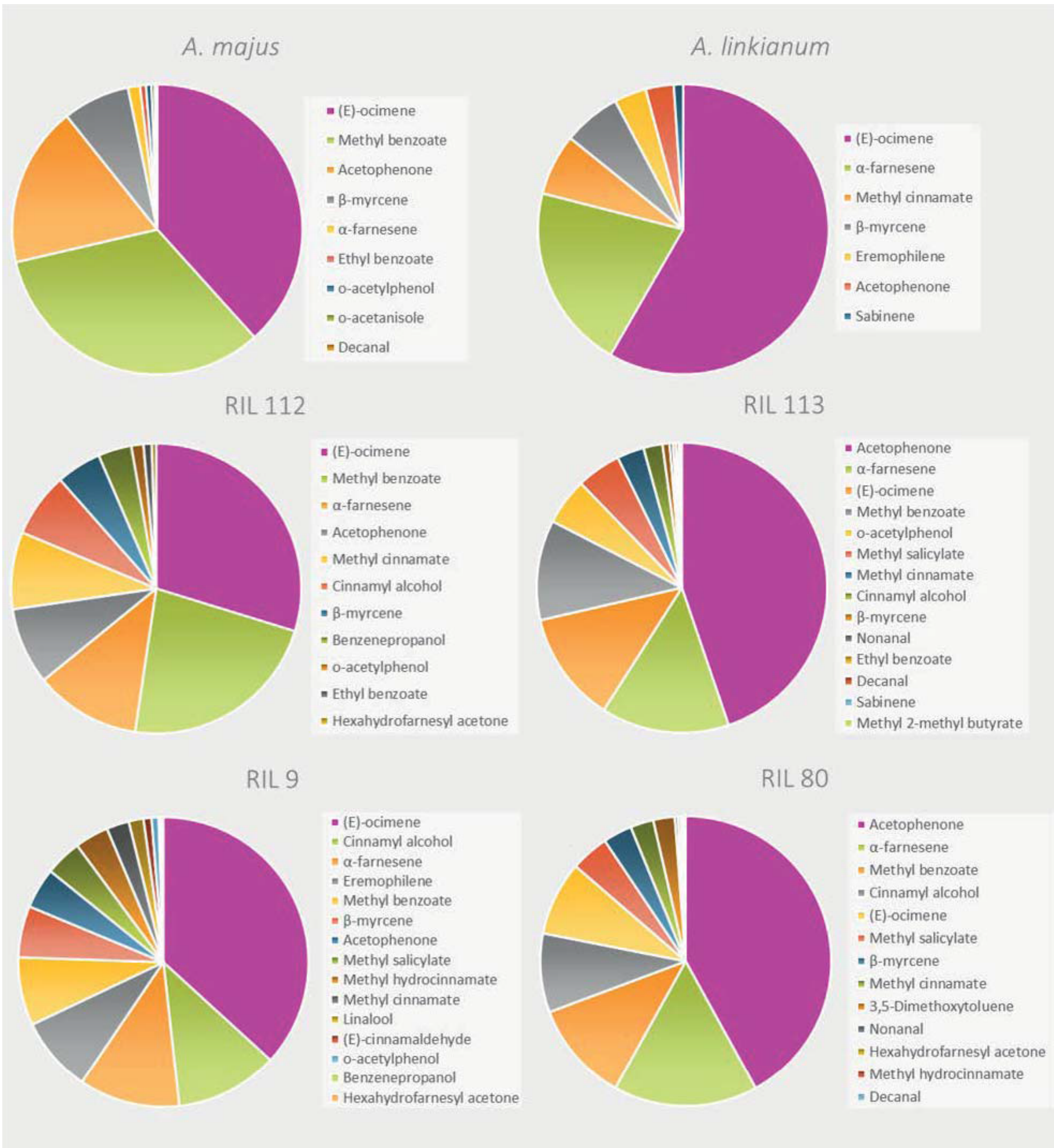

Figure 6. Scent profile components of *A. majus*, *A. linkianum*, RIL 112, RIL 113, RIL 9 and RIL 80. Pie charts order is according to relative quantities within scent profiles.

Minor VOCs found in RILs were, contrastingly, either shared with parental lines or emitted constitutively exclusively in RILs. Thus VOCs such as β myrcene, o acetylphenol, ethyl benzoate, methyl cinnamate, decanal, sabinene, (E) ocimene or acetophenone constituted part of the minor VOCs emitted in RILs 112, 113, 9 and 80 and were in common with those found in the scent profiles of *A. majus* and/or *A. linkianum*. However, some other compounds did not form part of the core volatile metabolome of parental lines. Hence VOCs such as cinnamyl alcohol, benzenepropanol, hexahydrofarnesyl acetone, methyl salicylate, nonanal, methyl 2 methyl butyrate, 3,5 dimethoxytoluene, methyl hydrocinnamate, linalool or (E) cinnamaldehyde were the products of the segregation of characters produced in RILs (Figure 6, Supplementary Table 1).

Figure 7 illustrates the differences or similarities regarding the emission of individual VOCs in each pairwise analysis (Figure 2). Different shades represent the degrees of correlation between lines. Hence, light green boxes indicate total dissimilarities, i.e. presence in line *A. majus* and absence in *A. linkianum* of o acetanisole. Contrastingly, magenta boxes indicate total similarities in the pairwise, i.e. absence in RIL 113 and absence in RIL 112 of o acetanisole (Figure 7). Intermediate shades indicate differences in quantities emitted of each compound in each pairwise.

Bumblebee preferences

Insects can be guided by attractant or repulsive responses to volatiles. There are two possible scenarios to pinpoint VOCs involved in their preferences. First, independent VOCs contrastingly and exclusively emitted in the pairwise with significant results may be the most plausible explanation. In this case, in Figure 7, light green boxes would appear in the pairwise and the rest of pairs would remain similar with green magenta shades. Second, combinations of VOCs can cause the choices of insects. These blends are more complex to be identified, however candidate VOCs, which can form part of them, can be recognised (Figure 7).

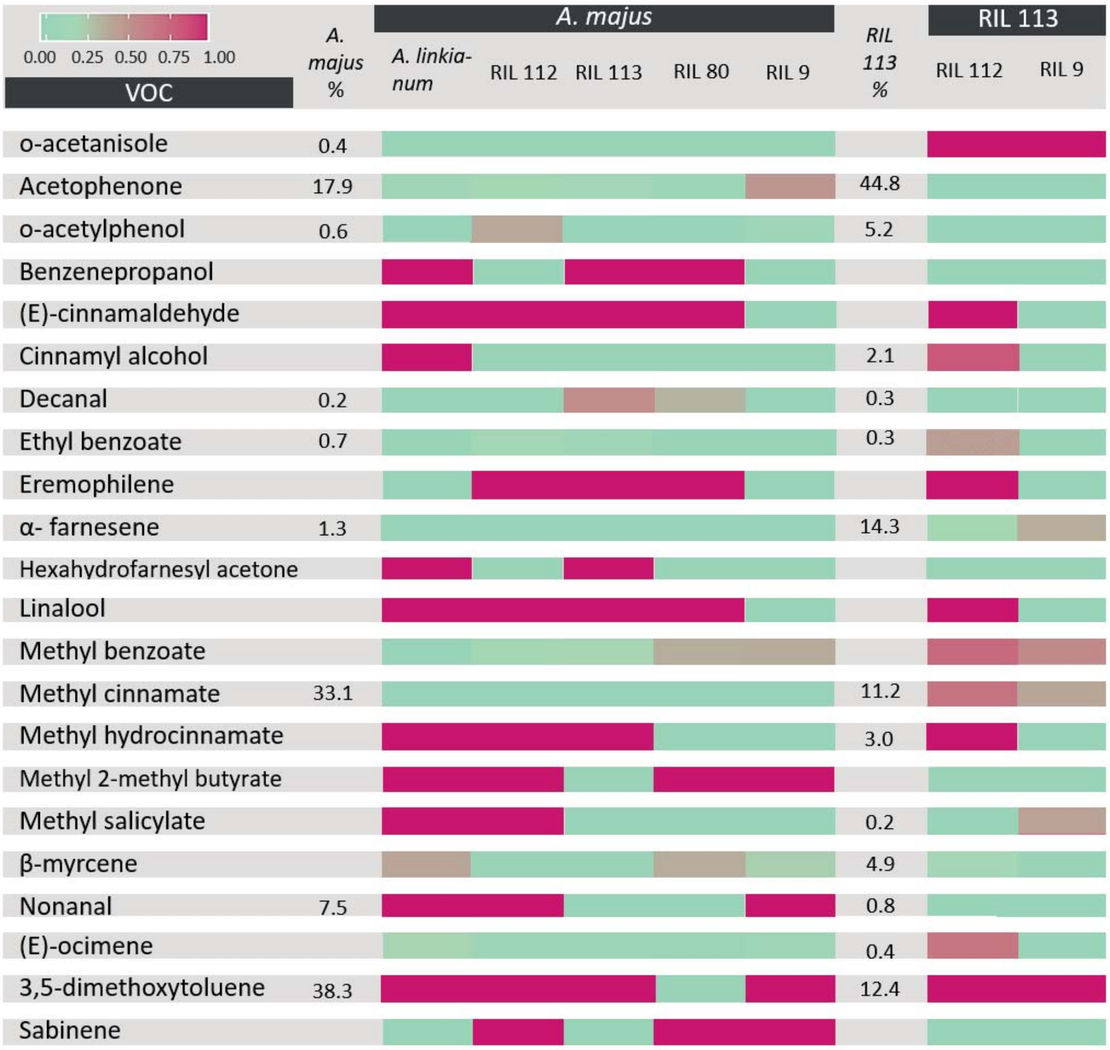

Figure 7. Dynamic range boxes indicating similarities in the emission of individual VOCs of *A. majus* versus *A. linkianum*, RIL 112, RIL 113, RIL 9 and RIL 80; and RIL 113 versus RIL 112 and RIL 9. Light-green boxes indicate diametrical differences between pairwise comparisons, while magenta boxes indicate total equality. To clarify, *A. majus* and RIL 113 percentage content of VOCs is indicated.

Considering quantities of scent emission, most scented lines (RILs 80 and 9) did not have an effect of attraction or repellence on bumblebees, as no preferences in pairwise *A. majus*   RIL 80, *A. majus*   RIL 9 or RIL 113   RIL 9, were found (Figure 5 and 8). Statistical significant results were found just for the pairwise *A. majus*   *A. linkianum* (p= 0.009). Between these lines, the only volatile which was exclusively emitted contrastingly in the pairwise was methyl benzoate. Methyl benzoate comprised a 33.1% of the total emission in *A. majus* whereas it was absent in *A. linkianum*. The rest of the lines (RILs) emitted different quantities of methyl benzoate, but these differences did not seem to have an effect on bumblebees' behaviour. Thus methyl benzoate appears to be a key volatile mediating bumblebee attraction (Figures 7 and 9, Supplementary Table 1).

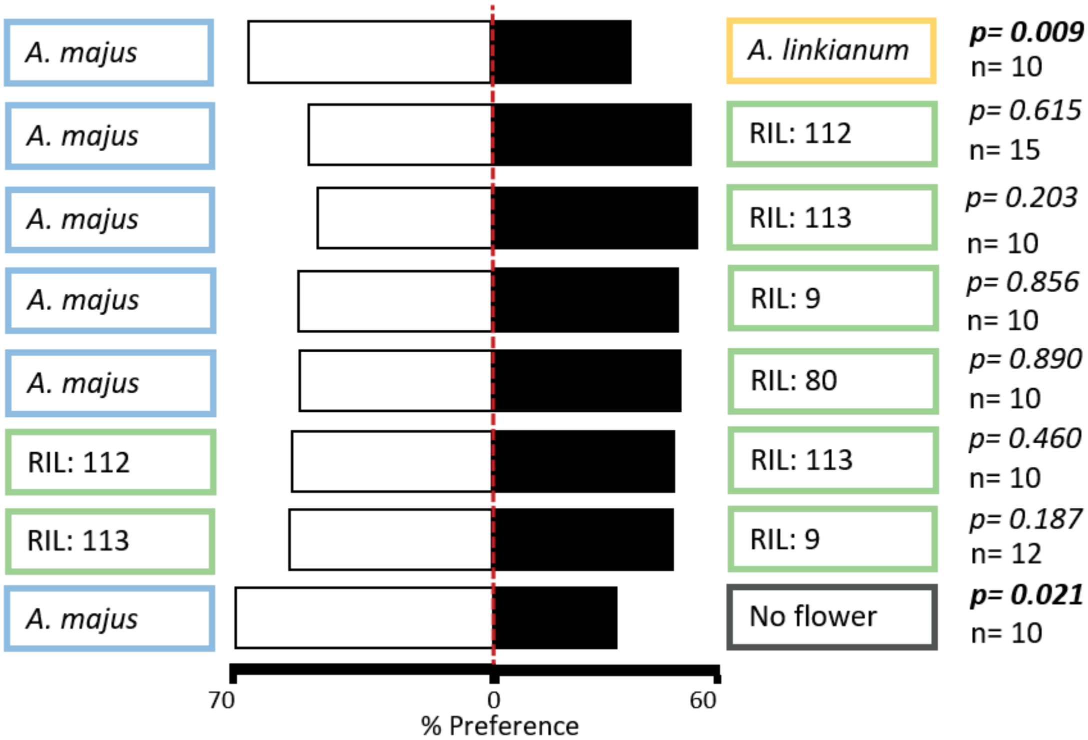

Figure 8. Bumblebee-preference tests between pairs. White and black boxes indicate the percentage of preference for either left or right line (indicated in colour boxes). Wilcoxon test p values and number of bees used, are indicated. Bold letters indicate statistical significant results.

Despite the apparent relevance of methyl benzoate on the behaviour of bumblebees, our results indicate a combinatorial effect of VOC blends on bee attraction towards *A. majus* against *A. linkianum*. Results also allow to pinpoint additional VOCs which can exert an effect of repellence of bumblebees from *A. linkianum* flowers. We found o acetanisole, decanal, ethyl benzoate and o acetylphenol which appear under the *A. majus* genotype but not in *A. linkianum* may enhance the attractive effect of *A. majus* against *A. linkianum*. Contrastingly, compounds emitted exclusively in *A. linkianum* when compared with *A. majus* may have an effect of bee repulsion, such as eremophylene, α farnesene, methyl cinnamate and sabinene (Figure 10a).

Independently from the preferences of bees for different scents, we tested the effect of floral scent as a cue (Figure 8). Results indicate that bumblebees are positively attracted to flowers aroma (p= 0.021). When they had to choose to feed either from floral scented places or non floral scented towers, they preferred to feed where there was a floral scent.

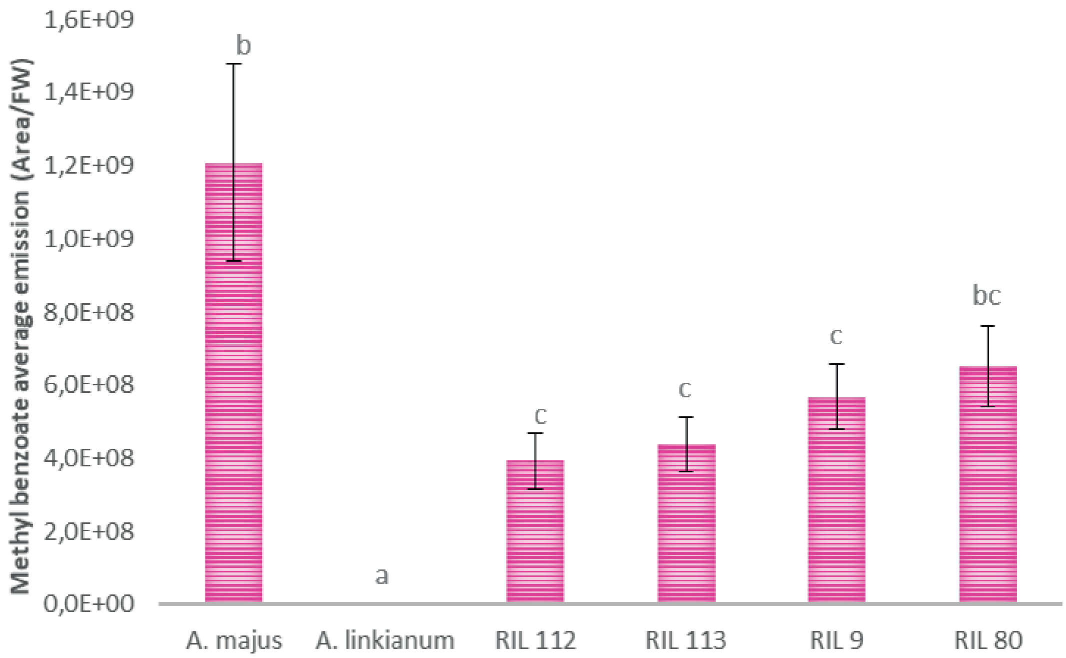

Figure 9. Methyl benzoate emission of *A. majus, A. linkianum*, RIL 112, RIL 113, RIL 9 and RIL 80. Letters indicate statistical significantly different results, according to Tukey HSD. Results are expressed as integrated areas by fresh weight (FW).

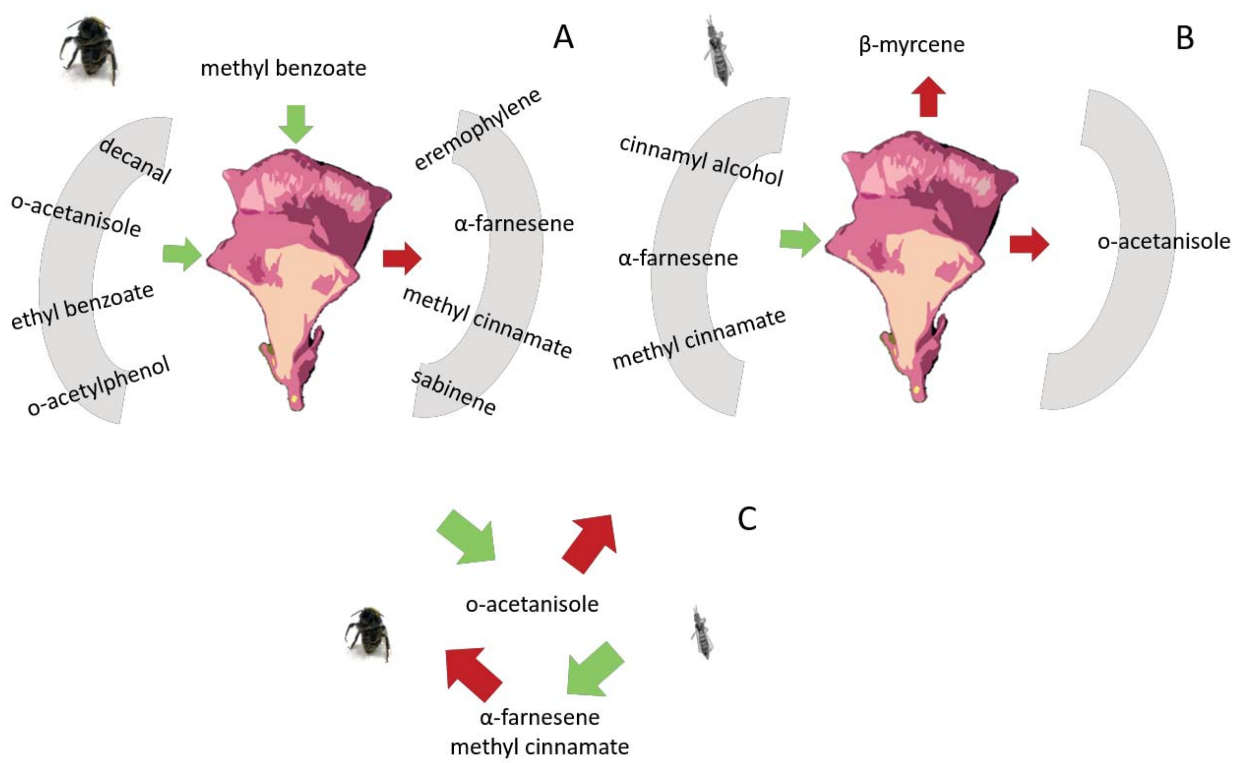

Figure 10. VOCs proposed to have an effect of attraction (green arrows) or repellence (red arrows) on bumblebees (A) and thrips (B). (C) VOCs with opposite effects on bumblebees and thrips. Left-side arrows refer to bumblebees and right-side arrows refer to thrips.

Bumblebee learning tests

Differential conditioning experiments indicated that despite the non preferences of bees for lines RIL 112 vs. RIL 113 and *A. majus* vs. RIL 9, they could differentiate their floral bouquets with p values < 0.001 ($z = 6.015$ and $z = 6.453$, respectively) (Figure 11). Bees began the experiments showing no preferences for quinine or sugar supplied towers, as they chose around a 50% of the time both kinds of towers. However bumblebee selections after 40 choices were between an 88 and 95% of the time to feed from sugar supplied towers (Table 2). Thus there is good evidence that bees learned to visit the correct flower based on scent alone. Control results indicated the inability of bees to distinguish between towers supplied with sugar or quinine and between black tape designs as they chose at the beginning and at the end of the experiment around a 50% of the time both kinds of towers ($z = 0.144$, $p = 0.885$). Therefore, bees did not learn which were rewarding towers and which were punishing towers under control experiment conditions (Figure 11, Table 2).

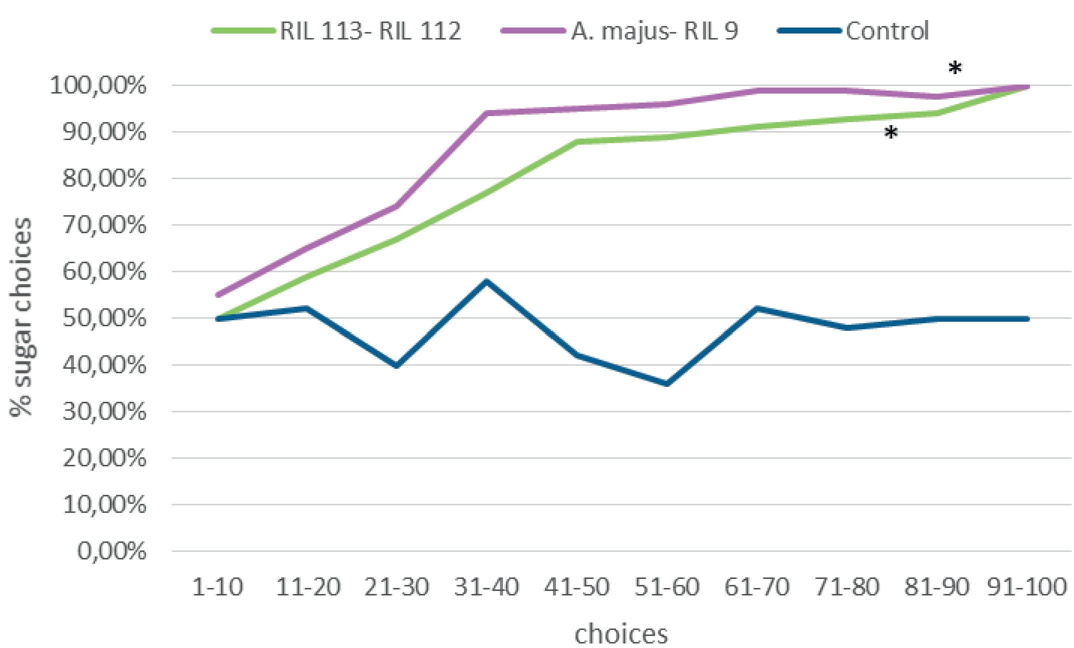

Figure 11. Percentage of choices of towers containing sugar as a reward against towers containing quinine as a punishment. Asterisks indicate significant statistical results and positive learning based on scent.

Table 2. Proportion of visits to towers supplemented with sugar as reward against quinine as punishment for the first and last ten choices. Pairwise analysis were performed using flowers from RIL 112 vs. 113 and flowers from *A. majus* vs. RIL 9. Control experiments were performed with flowers from the same line hidden inside towers supplemented with quinine as punishment and sugar as reward.

Pairwise	Choices 1-10	Choices 41-50	Choices 91-100
RIL 112- RIL113	50±18.9%	88±13.2%	-
A. majus - RIL 9	55±16.5%	95±7%	-
Control	50±12.3%	-	50±7.1%

Thrips preferences for flowers

The experimental conditions to test thrips flower preferences were adapted to a minute florivore, with direct contact with the flower. Quantities of scent emission did not seem to have an effect on the behaviour of thrips as they did not show a preference neither for high or low emitting lines under our experimental conditions (Figure 12).

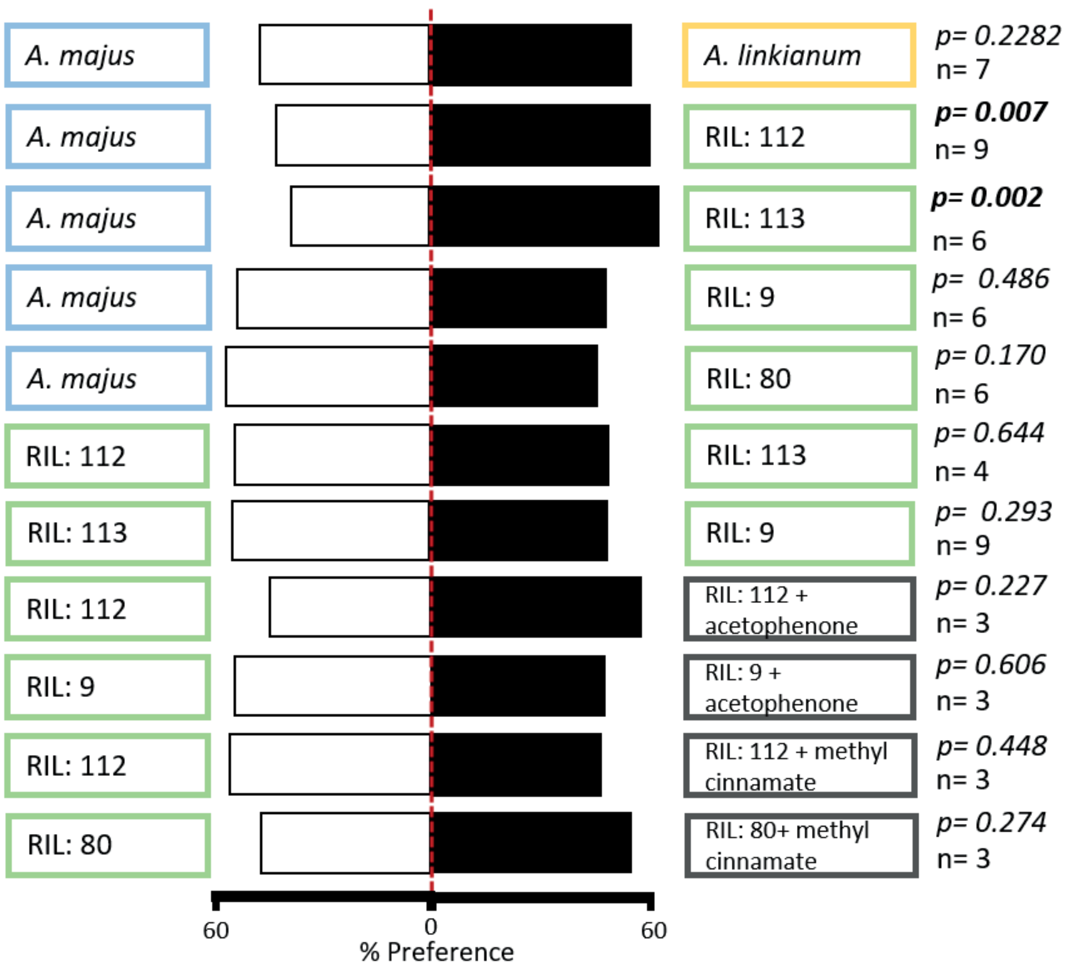

Figure 12. Thrips-preference tests between pairs and flowers from the same line supplemented with acetophenone or methyl cinnamate. White and black boxes indicate the percentage of preference for either left or right line (indicated in colour boxes). Chi square p-values and number of replicas, are indicated. Bold letters indicate statistical significant results.

Thrips were attracted by RILs 112 and 113 when compared with *A. majus* flowers (p: 0.007 and 0.002, respectively). Contrastingly, when these two RILs were confronted (RIL 112 113), thrips did not show preferences (p = 0.644) (Figure 12). By using dynamic range boxes we were able to pinpoint β myrcene as a volatile that differentiates RILs 112 and 113 from the rest of the lines (Figure 7). Deeper analysis indicated that the production of β myrcene in both RILs, 112 and 113, was 10 times lower than in *A. majus* flowers (Supplementary Table 1, Figure 13). Thus ß myrcene emerged as a candidate VOC to influence thrips preferences.

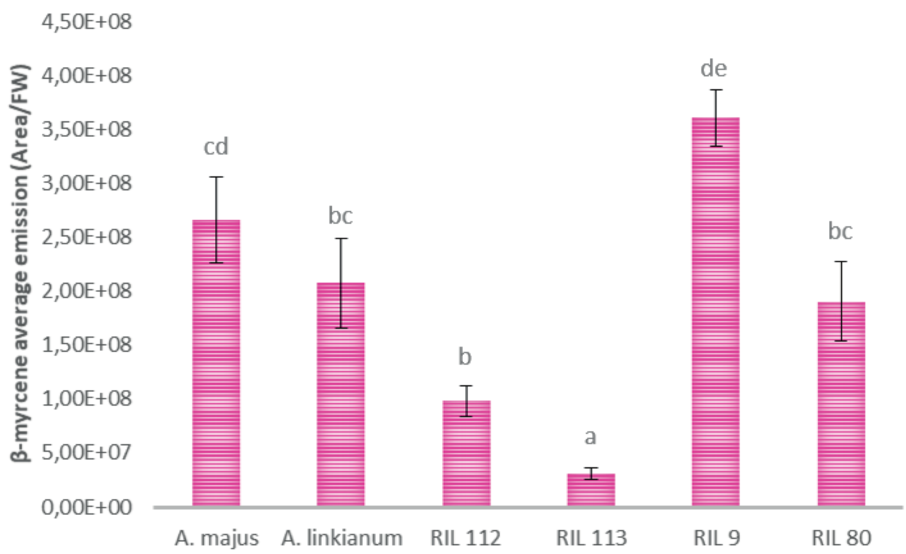

Figure 13. β-myrcene emission of *A. majus, A. linkianum,* RIL 112, RIL 113, RIL 9 and RIL 80. Letters indicate statistical significantly different results, according to t-test. Results are expressed as integrated areas by fresh weight (FW).

Nevertheless, results indicated that some additional VOCs apart from β myrcene may contribute to the interaction of flowers with thrips. These interactions can be of enhancement of either the repellence effect of high emitting β myrcene line *A. majus*, or the attraction towards low emitting β myrcene lines, RILs 112 and 113. Given that pairwise RIL 112 RIL 113 resulted in no preferences, combining VOCs can be assumed to be those in common between both lines. Acting as an enhancer of repellence but not as a key VOC with repulsive effects we found o acetanisole, as it is exclusively emitted in *A. majus*. Conversely, VOCs that may interact with thrips acting as attractants were cinnamyl alcohol and methyl cinnamate which were emitted constitutively in both RILs and not emitted in *A. majus*. Additionally, α farnesene was emitted at similar levels in both RILs (112: 11.8 %, 113:14.3 %) whereas it was emitted in much lower levels in *A. majus* (1.3 %). Thus α farnesene could be considered as a possible enhancer of the preferences of thrips for lines 112 and 113, acting as an attractant (Figures 6, 7 and 10b and Supplementary Table 1).

Effect of acetophenone and methyl cinnamate on thrips preferences

To test if the levels of acetophenone and methyl cinnamate were relevant for the preferences of thrips, we supplemented low emitting RILs of these VOCs to levels comparable in high emitting flowers. Afterwards, we tested the effect of supplemented flowers on thrips preferences. Flowers from RIL 112 and RIL 9 were supplemented with acetophenone and flowers from RIL 112 and RIL 80 were supplemented with methyl cinnamate. Under any circumstances, no significant values were found. Results indicate that increments on methyl cinnamate and acetophenone emissions do not have an effect of attraction or repellence on thrips under our experimental conditions (Figure 12).

Discussion

The interaction of plants with other organisms is partly controlled by the metabolome and volatilome acting as an interface for interactions. Regarding the evolution of flowering plants, positive selection is exerted on attractants and negative pressure is produced on deterrents by insects interacting with flowers. However, evidences show that the strongest selection is done by pollinators and not by herbivores (Bartkowska and Johnston, 2012; Farré Armengol et al., 2013).

Bumblebee behaviour mediated by floral scent

Previous studies in tomato showed that more scented flowers seemed to repel bumblebees, (Morse et al., 2012), while studies on blueberry under field conditions reported more visits in greater scented flowers (Rodriguez Saona et al., 2011). Contrastingly, our results indicate that composition of scent profiles rather than quantity of scent emission is the most important phenotypic trait for bumblebees, although these results should be tested in the field for corroboration. Independent of the quantities emitted, we show that bees prefer to feed from floral scented sites compared to simple sugar supply. Despite of being a signal used by bees to detect pollen and nectar in flowers (Knauer and Schiestl, 2015), floral scent seems to act as a cue for bumblebees to guide their choices.

Experiments testing the ability of bumblebees to differentiate scent profiles by associating a kind of smell to a reward or to a punishment, indicated that the lack of preferences of bumblebees for pairs tested is not caused by an inability to distinguish their scents. Instead it reinforces the no preferences results. This suggest that under field conditions, non preferred lines would plausibly be equally visited, in case just scent was mediating bumblebee choices.

Bumblebees and methyl benzoate

Out of the seven pairwise comparisons performed, bumblebees had significant preferences exclusively when *A. majus* was compared with *A. linkianum*. Instead, our results indicate that methyl benzoate may act as a key attractant within complex blends of VOCs tested. Methyl benzoate emission decreases after pollination both in *Antirrhinum* and Petunia (Negre, 2003), indicating a relevant role of this VOC on pollination. Further studies correlating methyl benzoate emission and quantities of rewards (nectar and pollen) may corroborate if this compound is an 'honest signal' for bumblebees (Johnson and Hobbhahn, 2010; Knauer and Schiestl, 2015).

Commercial breeding programs usually do not pay much attention to scent traits (Spiller et al., 2010). In the case of bumblebee pollinated crops, scent traits are broadly ignored for selection, even so the repulsive effects of tomato flower scent to bees has been reported for different commercially available tomato varieties (Whittington et al., 2004; Morse et al., 2012). Our results indicate that individual VOCs present in the flower bouquet can be related to more frequent visits of bumblebees and this observation may be relevant for the stimulation of pollination. In the case of methyl benzoate, breeding programs directed to the selection of emitting plants is a quite straightforward task, as the major gene promoting its ultimate synthesis, *BENZOIC ACID CARBOXYMETHYL TRANSFERASE* (*BAMT*) is already identified (Dudareva et al., 2000; Murfitt et al., 2000; Effmert et al., 2005).

A previous work showed that reduced emission of methyl benzoate in *A. linkianum* is related to rearrangements in the promoter region of the gene *BAMT*. Changes in the promoter region included an IDLE MITE transposon insertion when compared to the *A. majus BAMT* promoter region (Ruiz Hernández et al., 2017). Methyl benzoate is emitted by several *Antirrhinum* species in different proportions, including *A. majus* and *A. linkianum* (Weiss et al., 2016), despite the observed mutations in *BAMT*. This is caused by the fact that methyl benzoate is not exclusively synthesized by *BAMT* (Effmert et al., 2005). Our results reveal the profound effects that genetic changes affecting scent profiles might have on the evolution of species due to the loss of attractiveness of flowers to pollinators. In this sense, remarkable is the effect that transposon activities can have on genetic drift.

A. linkianum is a threatened species in Spain (Bañares et al., 2010), whereas *A. majus* is a widely distributed species (Vargas et al., 2009). The geographical isolation of *Antirrhinum* species was indicated as one of the reasons underlying the actual limited distribution of some of them (Vargas et al., 2009). In addition to the seclusion of populations, interactions with pollinators have also been proposed for mediating in *Antirrhinum* evolution (Glover and Martin, 1998; Vargas et al., 2009). The combination of habitat loss and the loss of a key attractant (methyl benzoate) of generalist pollinators such as bumblebees might have influenced deeply the threaten distribution of *A. linkianum* as its main pollinators are within hymenopters and lepidopterans (Comba et al., 1999). Our experiments were performed with the British subspecies *Bombus terrestris audax*, hence further experiments including natural occurring pollinators of *A. linkianum* and *A. majus* may bring to light additional knowledge on the subject (Ings et al., 2009; Vargas et al., 2009).

Thrips behaviour regarding β-myrcene, acetophenone and methyl cinnamate

The way thrips preferences for floral scents were assessed here did not allow us to isolate scent traits from other floral traits such as colour, size or shape. Nevertheless, we have been able to pinpoint an important volatile mediating thrips preferences, β myrcene. This compound is constitutively emitted by any of the lines studied, however, just two lines emit it in significantly lower levels: RIL 112 and RIL 113. These lines were significantly preferred by thrips, indicating a possible effect of deterrence of this compound. Volatilomes have been described as an interface that equilibrates the attraction of mutualists and the repellence of antagonists in plants (Farré Armengol et al., 2013; Ceuppens et al., 2015). Therefore constitutive compounds emitted by flowers may act as repellents and a decrease in the emission of individual VOCs may decrease the repellence effect, as our results indicate. Changes in relative proportions of VOCs within scent profiles have been described to interact as signals for insects (Rodriguez Saona et al., 2011; Farré Armengol et al., 2013). For instance, thrips *Cycadothrips chadwicki* (Mound) is the exclusive pollinator of some *Macrozamia* cycad species. It has been reported the correlation between increases in the level of emission of β myrcene and the leave *en masse* of thrips from cones, however β myrcene increase is associated to the thermogenesis event during a specific time of the day (Terry et al., 2007; Terry et al., 2014). Our results are concordant with numerous studies which indicate a repellence activity of β myrcene on mosquitoes, beetles and cockroaches (Hwang et al., 1985; Yoon et al., 2007; Yoon et al., 2009). Altogether evidences indicate a repellence effect on thrips of β myrcene, although redundant, complementary, or synergistic signals may play a role (Raguso and Willis, 2002)

Breeding programs should incorporate knowledge on attractants and repellents of thrips in order to increase plant resistance and diminish pesticide usage. Two myrcene synthases (*ama1e20* and *ama0c15*) are known in snapdragon (Dudareva et al., 2003). Changes in quantities of β myrcene emission might be related to allelic mutations in these genes and could be further explored in the context of pest control.

Additionally, we assessed the effect of acetophenone and methyl cinnamate increases on thrips behaviour. Flowers supplemented with acetophenone did not have an effect on the comportment of thrips, despite that it has been described the repellence effect of acetophenone on bumblebees and beetles (Erbilgin et al., 2008; Suchet et al., 2010). Regarding methyl cinnamate, we found that analysis supplementing low emitting flowers with it did not have an effect on thrips preferences. This result refused our hypothesis that it could be an attractant for thrips acting as part of blends. However, subsequent tests assessing effects of quantities may bring to light different results.

Scent blends mediating in the attraction/repellence of bumblebees and thrips

Results indicate that in addition to the segregation of ocimene, methyl benzoate, acetophenone and methyl cinnamate (Ruiz Hernández et al., 2017), transcription factors which activate the synthesis of VOCs non constitutively emitted in parental lines segregated in the RILs. Hence, further genomic studies using these lines may reveal transcription factors involved in the emission of VOCs such as cinnamyl alcohol, nonanal or methyl salycilate (Yu et al., 2001; Lu et al., 2016). Remarkably, except for cinnamyl alcohol, none of the VOCs found exclusively in RILs showed an important role in the interaction with thrips and bumblebees, indicating that in wild types constrains have been exerted against these VOCs.

Although we were able to highlight compounds as key VOCs influencing the behaviour of bees and thrips, our results also indicate a combinatorial effect of VOC compounds on insect comportment. Numerous scent blends have been described for having an effect on insects (Takabayashi et al., 1991; Douglas et al., 2004; Yuan et al., 2008; Rodriguez Saona et al., 2011). Remarkably, our data indicate that o acetanisole seems to attract bees and repel thrips when acting in combination (Figure 10c). o acetanisole has been described for having an insecticide effect against maize weevils (*Sitophilus zeamais*) and booklice (*Liposcelis bostrychophila*) (Quan et al., 2018), however no previous studies refer to its implication on pollination. Furthermore, α farnesene and methyl cinnamate may act in blends as repellent to bumblebees and attractant to thrips (Figure 10c). Our results match with previous studies indicating the attractive dose dependent effect of (E) β farnesene on thrips (Manjunatha et al., 1998; Koschier et al., 2000). However there is discrepancy with previous studies that pinpointed (E) α farnesene as a private channel attracting bumblebees (Knauer and Schiestl, 2017). Our results contrast with the general association of the common floral volatile methyl cinnamate as an attractant to pollinators (Schiestl and Roubik, 2003), even so a repellent effect on insects has also been described (Sawahata et al., 2008).

Furthermore, bumblebees seem to be attracted to blends containing decanal, o acetanisole, ethyl benzoate and o acetylphenol. To our knowledge, the fatty acid derivative decanal has not been previously indicated as mediating bumblebees behaviour. However, reports indicate its effect mediating plant insect interactions as part of mixtures of VOCs (Douglas et al., 2004; Yuan et al., 2008). On the other hand, bees might be attracted by scent mixtures including ethyl benzoate, which mediates the interactions of plants with insects (Takabayashi et al., 1991) and as part of a blend, attracts orangeworm moths to almond trees (Beck et al., 2012). Regarding o acetylphenol, its substitution p acetylphenol is involved in juvenile hormone and acaricide activities (Massardo et al., 1978), however no reports indicate any effect on bumblebees. Additionally, distinct terpenes may have repellence effects on bumblebees like sabinene which is known for having an antifungal activity (Kohzaki et al., 2009), and eremophilene which is involved in plant defence in eucalyptus (Troncoso et al., 2011).

In addition, thrips might be also attracted by cinnamyl alcohol. This compound has been proposed for mediating on blueberry pollination status indications to pollinators (Rodriguez Saona et al., 2011), however it has not been previously indicated as mediating in thrips flowers interactions.

Our results concur with the general association of benzenoids to pollinator attraction. These compounds are phenylpropanoids related to floral pigments, (van Schie et al., 2006; Farré Armengol et al., 2013). Phenylpropanoids, such as methyl benzoate, ethyl benzoate and benzaldehyde, are generalist pollinator attractants (Johnson and Hobbhahn, 2010). Contrastingly terpenoids such as eremophylene, farnesene or sabinene, are usually found in vegetative tissues with repellent activities. Although the abundant terpene family harbours both attractants and deterrents (Farré Armengol et al., 2013).

Further experiments with male thrips and bumblebees may yield different results as scent preferences and the ability to detect aromas have been proved to depend on genre in some insects, as experiments with *F. occidentalis*, *Lygus hesperus* and *Aegorhinus superciliosus* indicate (Blackmer and Ca As; Parra et al., 2009; Cao et al., 2018). No scent oriented experiments have been done on this subject on bumblebees. However, regarding visual floral cues, male and female bumblebees do not differ in their learning speed nor their ability to overcome previously acquired associations (Wolf and Chittka, 2016).

Conclusions

Our results indicate that both scents emission of discrete elements and blends are under pressure for selection in flowers by pests and pollinators. On one side, methyl benzoate attracts bumblebees and quantities emitted are not relevant for this effect. In addition, it does not have an effect on thrips. Moreover, β myrcene repels thrips but not bumblebees and changes in its concentrations are determinant in affecting thrips behaviour. β myrcene and methyl benzoate effects may be enhanced or diminished by combinations of VOCs. Compounds proposed to form part of blends with attracting or deterrent effects, could be taken into account in further studies on the behaviour of insects. Breeding programs could include the selection of methyl benzoate and β myrcene synthases coding genes, in order to increase fitness in commercial varieties. As shown in this work, multivariate analysis of scents can return valuable agronomical knowledge. However, redundant, complementary, or synergistic relation between olfactive and other floral traits should be explored.

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Supplementary material

Supplementary table 1. VOCs content in *A. majus, A. linkianum* and RILs 112, 113, 80 and 9, expressed in percentage (%) and as integrated peak areas by fresh weight (FW).

VOC	*A. majus*		*A. linkianum*		RIL 112		RIL 113		RIL 80		RIL 9	
	%	Peak area/FW	%	Peak area/FW	%	Peak area/FW	%	Peak area/FW	%	Peak area/FW	%	Peak area/FW
o-acetanisole	0.4	1.60E+07										
Acetophenone	17.9	6.46E+08	3.1	1.06E+08	8.6	1.55E+08	44.8	1.80E+09	42.0	2.41E+09	4.5	3.56E+08
o-acetylphenol	0.6	2.52E+07			1.3	2.51E+07	5.2	2.14E+08			0.8	5.87E+07
Benzenepropanol					3.7	6.03E+07					0.3	1.63E+07
(E)-cinnamaldehyde											0.9	6.07E+07
Cinnamyl alcohol					7.2	1.17E+08	2.1	8.66E+07	8.8	4.38E+08	11.4	7.48E+08
Decanal	0.2	9.15E+06					0.3	1.25E+07	0.3	1.56E+07		
Ethyl benzoate	0.7	2.41E+07			0.9	1.67E+07	0.3	1.23E+07				
Eremophilene			3.6	1.15E+08							8.4	5.79E+08
α-farnesene	1.3	5.00E+07	20.7	5.93E+08	11.8	2.06E+08	14.3	5.82E+08	16.0	1.05E+09	11.2	8.78E+08
Hexahydrofarnesyl acetone					0.5	8.99E+06			0.3	1.91E+07	0.2	1.77E+07
Linalool											1.6	1.09E+08
Methyl benzoate	33.1	1.21E+09			22.6	3.91E+08	11.2	4.38E+08	11.3	6.51E+08	7.6	5.68E+08
Methyl cinnamate			6.8	2.42E+08	8.6	1.52E+08	3.0	1.24E+08	2.6	1.38E+08	2.5	1.21E+08
Methyl hydrocinnamate									0.3	1.48E+07	3.8	1.75E+08
Methyl 2-methyl butyrate							0.2	5.91E+06				
Methyl salicylate							4.9	2.07E+08	4.2	2.62E+08	4.1	3.13E+08
β-myrcene	7.5	2.66E+08	6.4	2.07E+08	5.0	9.60E+07	0.8	3.07E+07	3.2	1.91E+08	5.7	3.61E+08
Nonanal							0.4	1.26E+07	0.3	1.84E+07		
(E)-ocimene	38.3	1.37E+09	58.3	1.72E+09	29.7	5.65E+08	12.4	4.96E+08	8.3	5.17E+08	33.2	2.14E+09
3.5-dimethoxytoluene									2.4	1.37E+08		
Sabinene			1.0	3.39E+07			0.2	6.31E+06				

Conclusions

The study of the genetic basis of scents emission from plants relies on detailed and comparative phenotyping and genotyping. *Antirrhinum* has served as a model plant for the study of floral volatiles from several points of view. We analysed the scent profiles of several wild species and two laboratory lines. Furthermore, we approached the genetic basis of the differences between *A. majus* and *A. linkianum.* With regard to these two species, we studied the effect of their contrasting floral scent profiles on pests and pollinators. Additionally, we provide a discussion of the methodologies available for quantifying VOCs and argue their suitability. Finally, we developed a tool to ease the analysis of GC MS data which can allow the increment of samples analysed and increase the robustness of studies on phenotypic spaces.

Each chapter of this PhD thesis provides a series of conclusions that deepen our knowledge on both *Antirrhinum* floral scents and the study of scents emission in general.

Chapter I

∴ There is a broad chemodiversity in scent emission in *Antirrhinum*.

∴ In flowers, the developmental stage plays an important role in the quantities emitted of VOCs.

∴ The relative robustness of floral bouquets in *Antirrhinum* could be an adaptation to local pollinators.

∴ majus Sippe 50 and A. braun blanquetii, showed development specific changes in their VOC composition, suggesting a precise control and fine tuning of scent profile

Chapter II

∴ The contrasting scent emission of A. majus and A. linkianum can be explained for each compound by a simple Mendelian segregating model based on one or two genes.

∴ The lack of emission of methyl benzoate in A. linkianum is a result of major rearrangements in the promoter region of BAMT.

∴ Natural variation of scent profiles may be the result of combinations of wild type and loss of function alleles in genes involved in discrete VOCs biosynthesis.

∴ Active transposable elements in the genus may account for quick evolution and instability of species characteristic scent traits.

Chapter III

∴ In sets of samples with a high biological variability, as in case of flowers, the semi quantification by standard addition is not a feasible method.

∴ Any of the methodologies presented can be considered as valid for scent profiling as they all gave highly similar statistical results: external calibration curves generated in the sampling system and by liquid addition of standards to stir bars (in any case, irrespective of the usage of nearest components and single calibrators for semi quantifications), total integrated peak area per fresh weight (FW), normalized peak area per FW, semi quantification based on internal standard abundance, semi quantification based on the nearest n alkane and percentage of emission.

∴ For the study of relative proportions of VOCs, the generation of calibration curves for each compound analysed is not necessary.

∴ From our point of view and concerning the plant scent community, a general methodological consensus would be desirable in order to ease the comparison of data.

Chapter IV

∴ gcProfileMakeR provides a fast and reliable means of analysing GC MS non targeted data, and should help in comparative metabolome studies of genotypes and/or environmental samples.

∴ gcProfileMakeR allows to separate into groups VOCs which can form part of the core volatile metabolome and VOCs considered as non constitutive, either for lack of quality of the matching with MS libraries or for insufficient frequency to be considered as constitutive.

∴ Working with CAS numbers gcProfileMakeR enhances reproducibility and FAIR data implementation.

Chapter V

∴ Methyl benzoate attracts bumblebees and quantities emitted are not relevant for this effect. Additionally, methyl benzoate does not have an effect on thrips.

∴ β myrcene repels thrips but not bumblebees and changes in its concentrations are determinant in affecting thrips behaviour.

∴ β myrcene and methyl benzoate effects may be enhanced or diminished by combinations with additional VOCs, forming scent blends.

- ∴ Compounds proposed to form part of blends could be taken into account in further studies on the behaviour of insects.
- ∴ Breeding programs could include the selection of methyl benzoate and β myrcene synthases coding genes, in order to increase fitness in commercial varieties.
- ∴ Redundant, complementary, or synergistic relation between olfactive and other floral traits should be explored.

General conclusion

This piece of research has addressed several important biological and technical questions in the field of plant volatiles. Research conducted has ranged from analytical chemistry to genetics, ecology and agronomy. On the biological side, we have provided insights in the study of *Antirrhinum* scent profiles and their effects on pests and pollinators. These works allowed us to study the genetic and evolutionary implications of volatile emission, but they also delivered some knowledge which can easily be applied in the field of agronomy. On the technical side, we provide methodologies and tools which can serve to a wider research community.

Conference communications

XIV REUNIÓN DE BIOLOGÍA MOLECULAR DE PLANTAS

SALAMANCA, 4-6 de Julio 2018

MAJOR PHENOTYPIC TRAITS UNDER SELECTIVE PRESSURE IN *ANTIRRHINUM*

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Plant aromas are composed by independent Volatile Organic Compounds (VOCs) in different proportions. Scent profiles are distinctive blends that can characterize species as has been published for *Antirrhinum* (Weiss et al. 2016). The genetic structure of the emission of independent VOCs in this genus appears to be composed of single genes coding for VOC synthesis enzymes (Ruiz Hernández et al. 2017). We developed a recombinant inbred line of *A.majus* x *linkianum*, and found segregating lines for ocimene, methyl benzoate, methyl cinnamate and acetophenone. We have assessed preferences of pest *Frankliniella occidentalis* and pollinator *Bombus terrestris* for different floral scent profiles. Our results indicate that changes in scent profiles have an important role in pest and pollinator choices, therefore having an impact on natural and agricultural ecosystems.

Major phenotypic traits under selective pressure in *Antirrhinum*

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Background

- Plant aromas are composed by independent Volatile Organic Compounds (VOCs) in different proportions. Scent profiles are distinctive blends that can characterize species as has been published for *Antirrhinum* (Weiss et al. 2016).
- The genetic structure of the emission of independent VOCs in this genus appears to be composed of single genes coding for VOC synthesis enzymes (Ruiz-Hernández et al. 2017).
- We developed a recombinant inbred lines of *A. majus* x *A. linkianum*. We have assessed preferences of pest Frankliniella occidentalis and pollinator Bombus terrestris for different floral scent profiles. Our results indicate that changes in scent profiles have an important role in pest and pollinator choices, therefore having an impact on natural and agricultural ecosystems.

Scent phenotypes

We used *A. majus, A. linkianum* and four recombinant imbred lines, RILs (Figure 1). We have established the constitutive scent profile of each line. Core scent profiles of different lines varied in the number and quantities of VOCs emitted (Figure 2). Parental lines floral scent profiles were less complex than those of their descendants.

Figure 1. *Antirrhinum* flowers from *A. majus, A. linkianum*, RIL 112, RIL 113, RIL 9 and RIL 80.

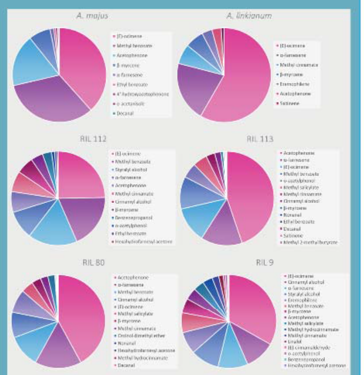

Figure 2. Scent profile components of *A. majus, A. linkianum*, RIL 112, RIL 113, RIL 9 and RIL 80. Pie charts order is according to relative quantities within scent profiles.

Similarities and differences in VOCs emission

Parental lines produced constitutively 12 out of the total amount of 24 VOCs identified. Independent VOCs contrastingly and exclusively emitted in the pairwise with significant results, may be the most plausible explanation for the preferences of insects (Figure 3).

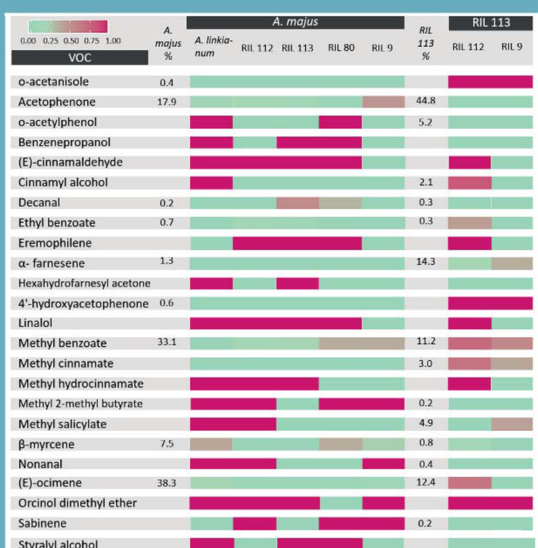

Figure 3. Dynamic range boxes (dynRB package) indicating similarities in the emission of individual VOCs of A. majus versus A. linkianum, RIL 112, RIL 113, RIL 9 and RIL 80; and RIL 113 versus RIL 112 and RIL 9. Light-green boxes indicate diametrical differences between pairwise comparisons, while magenta boxes indicate total equality. To clarify, A. majus and RIL 113 percentage content of VOCs is indicated.

Bumblebees preferences

Regarding bumblebee preferences for floral scents (Figure 4), statistical significant results were found just for the pairwise *A. majus - A. linkianum* (p= 0.021). Between these lines, the only volatile which is exclusively emitted contrastingly in the pairwise is **methyl benzoate**. *A. majus* emits methyl benzoate in a 33.1%, whereas *A. linkianum* does not emit it. The rest of the lines emit methyl benzoate, with different quantities which did not have an effect on bumblebees behaviour (Figure 5).

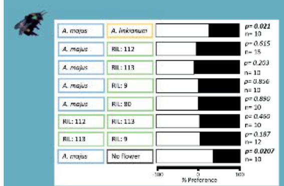

Figure 4. Bumblebee-preference tests between pairs. Wilcoxon test p values and number of bees used, are indicated. Bold letters indicate statistical significant results.

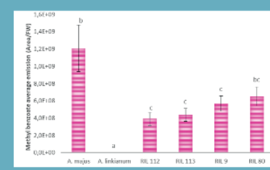

Figure 5. Methyl benzoate emission of *A. majus, A. linkianum*, RIL 112, RIL 113, RIL 9 and RIL 80 Letters indicate statistical significantly different results, according to Tukey HSD. Results are expressed as integrated areas by fresh weight (FW).

Thrips preferences

Thrips are attracted by RILs 112 and 113 when compared with *A. majus* flowers. Contrastingly, when these two RILs are confronted (RIL 112 - 113), thrips do not prefer any of them (Figure 6). In this case, **β-myrcene** is produced 10 times less in lines 112 and 113 than in the rest of the lines (Figure 7) which leads to significant differences, making it a candidate to influence thrips preferences.

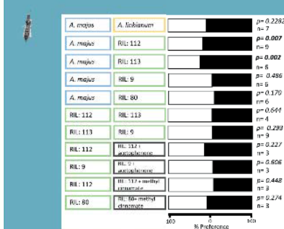

Figure 6. Thrips preference tests between pairs and flowers from the same line supplemented with acetophenone or methyl cinnamate. Chi square p values and number of replicas, are indicated. Bold letters indicate statistical significant results.

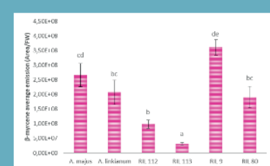

Figure 7. β-myrcene emission of *A. majus, A. linkianum*, RIL 112, RIL 113, RIL 9 and RIL 80. Letters indicate statistical significantly different results, according to t test. Results are expressed as integrated areas by fresh weight (FW).

Conclusions

Scent is an important phenotype under selection for pests and pollinators:

- **β-myrcene repels thrips** but not bumblebees and changes in its concentrations are determinant in affecting thrips behaviour.
- **Methyl benzoate attracts bumblebees** and quantities emitted are not relevant for this effect. Additionally, methyl benzoate does not have an effect on thrips.
- **Breeding programs** could include the selection of methyl benzoate and β-myrcene synthases coding genes, in order to increase fitness in commercial varieties.
- Mixtures of volatiles with effects over insects can also be indicated from our study. These blends could be taken into account in further studies on the behaviour of insects.

References

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NORWICH, 24–26 July 2017

THE EFFECT OF MODIFIED SCENT PROFILES ON PEST AND POLLINATOR CHOICES

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Recent publications have shown the contrasting floral scent profiles of the laboratory inbred line 165E of *Antirrhinum majus* and a complete set of *Antirrhinum* wild species. The molecular phylogenetic markers and the botanical phylogeny coincide with the different scent profiles analysed. We constructed recombinant inbred lines between *A. majus* 165E and *A. linkianum* differing in the emission of methyl benzoate, methyl cinnamate, acetophenone and ocimene. We have analysed the genetic structure of scent emission in these RILs. We cloned a loss of function allele of BENZOIC ACID CARBOXYMETHYL TRANSFERASE. The recombinant inbred lines have been used to test attraction and repulsion of the pest *Frankliniella occidentalis* and pollinator *Bombus terrestris*. Our results indicate an evolutionary pressure and/or drift towards distinct scent profiles in the different species. Scent profiles display mendelian segregations linked to single scent components. The null allele of *A.linkBAMT* is caused by a major rearrangement in the promoter including an IDLE MITE transposon insertion. Both thrips and bumblebees show distinct preferences for floral scent blends. Complex scent profiles may be achieved by combinations of genes that are dynamically changing as a result of transposons. Changes in scent profiles may have profound effects on pest and pollinator choices.

The effect of modified scent profiles on pest and pollinator choices

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Background

- Scent and volatiles are an interface driving interaction between plants and a large array of organisms such as bacteria, fungi, herbivores or pollinators. Floral scent is formed by combinations of Volatile Organic Compounds (VOCs).
- We have used the *Antirrhinum* genus as a model to study the genetic structure and evolution of scent as it comprises over 25 species with a history of intercrossing and evolution back to a parental type. Analysis of floral scent in eight *Antirrhinum* wild species and two *A. majus* lines identified 130 compounds such as phenylpropanoids, benzenoids, mono- and sesquiterpenes, nitrogen-containing compounds and aliphatic alcohols.
- Complex scent profiles may vary as a result of simple or epistatic segregations. The effect of transposable elements over phenotypes can be determinant (Rabbani and Wahl, 2016), as well as, the effect that these phenotypic changes have over other organisms preferences and species evolution.

Botanical phylogeny matches floral scent profiles

Using the volatile profiles we were able to construct the phylogenetic subsections of the *Antirrhinum* genus (Weiss et al., 2016).

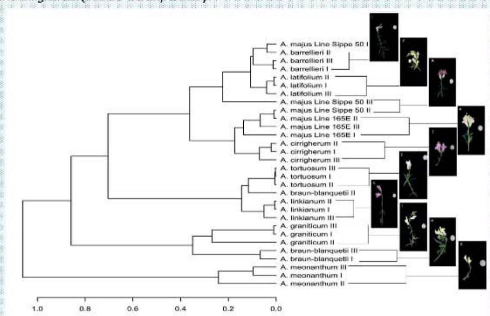

Figure 1. Cluster analysis based on scent profiles of the different *Antirrhinum* species sampled at different flower developmental stages. Pictures show flowers from the studied species

Mendelian segregations

We performed a cross between *A. majus* and *A. linkianum*. These species have contrasting emissions of methyl benzoate, β-ocimene, methyl cinnamate and acetophenone. We found that these selected compounds displayed mendelian segregations on F2 plants (Ruiz-Henández et al., 2017).

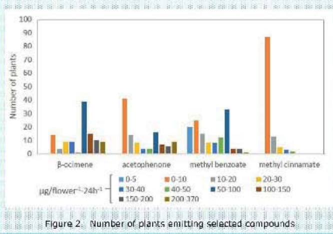

Figure 2. Number of plants emitting selected compounds

Table 1. Statistical analysis of the Mendelian segregations in a F2 population of selected compounds (n = 110 plants)

Compound	Segregation model	Chi square P-value
Methyl benzoate	3:1 (85:25)	0.582
β-Ocimene	3:1 (96:14)	0.002953
	3:1 (92:18)	0.03645
	3:1 (83: 27)	0.9123
	13:3 (96:14)	0.1056
Methyl cinnamate	3:1 (87:23)	0.3218
Acetophenone	3:1 (68:41)	0.002354
	1:2:1 (41:46:22)	0.0001789
	9:7 (68:41)	0.1966

BAMT promoter

We amplified and sequenced 2131 bp of the promoter region of *A. majus*, and compared it to the corresponding fragment of the *A. linkianum* genome. We found a set of rearrangements in *A. linkianum* promoter, including two major insertions with homologies with an Idle-MITE transposable element, a fragment found in the promoter region of the PLENA locus (BLASTN e-114) and the VENOSA genomic locus (BLASTN 2e-19). Our findings indicate that these changes at the promoter level in *AlBAMT* cause the loss of function of the gene in *A. linkianum* (Ruiz-Hernández et al. 2017).

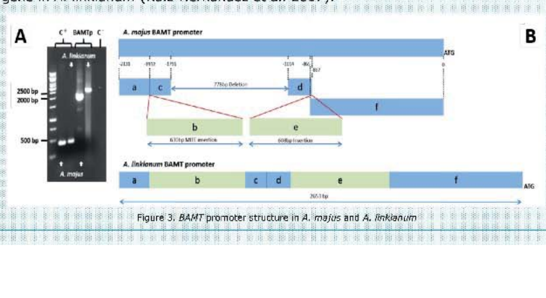

Figure 3. *BAMT* promoter structure in *A. majus* and *A. linkianum*

Recombinant scent profiles

We selected four recombinant inbred lines (RILs) with contrasting floral scent profiles regarding major VOCs emission. We have used these RILs plus the parental species flowers to asses pest and pollinator preferences.

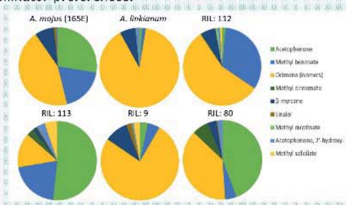

Figure 4. Percentage of emission of major compounds in *A. majus* (165E), *A. linkianum and RILs: 112, 113, 9 and 80*

Thrips and bumblebees preferences

We have performed experiments evaluating *Frankliniella occidentalis* and *Bombus terrestris audax* preferences. Experiments have been carried out in pairwises. Thrips experiments were done by using full flowers without stamen and stamen separately. Bumblebee experiments assessed preferences by scent (hiding flowers but allowing scent release) and by using full flowers. **Results show that changes in scent profiles in RILs comparing to their parental species significantly affect bumblebee and thrips preferences. *A. majus* and *A. linkianum* are attractive for pollinator and unattractive for pest, whereas their offspring have lost their selective advantage**. Except line 112 that is preferred by both pest and pollinator. Major VOCs do not seem to have a direct effect over pest and pollinator choices. Further analysis of VOCs emitted on a subtle fashion is needed, as well as additional phenotypic traits.

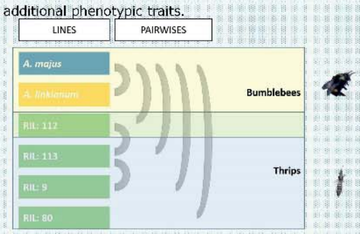

Figure 5. Summary results of preferences from bumblebees (yellow shaded) and thrips (blue shaded). Grey line indicate the pairwises assessed

Conclusions

Our data shows that genome evolution and traits under apparently strong evolutionary pressure may be subject to rapid changes as a result of transposon-mediated small-scale insertions and deletions.

Changes in key phenotypes might affect pest and pollinator choices thus having an effect over survival and fertility. Scent is an important phenotype under selection for pests and pollinators. Our future results will elucidate the importance of this trait comparing to others like colour, size...

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Weiss J, Mühlemann JK, Ruiz-Hernández V, Dudareva N, Egea-Cortines M: **Phenotypic space and variation of floral scent profiles during late flower development in Antirrhinum**. *Front Plant Sci* 2016, **7**:1903.

Ruiz-Hernández V, Hermans B, Weiss J, Egea-Cortines M: **Genetic Analysis of Natural Variation in Antirrhinum Scent Profiles Identifies BENZOIC ACID CARBOXYMETHYL TRANSFERASE As the Major Locus Controlling Methyl Benzoate Synthesis**. *Front Plant Sci* 2017, **8**:27.

XIII REUNIÓN DE BIOLOGÍA MOLECULAR DE PLANTAS

OVIEDO, 22-24 Junio 2016

COMPLEX FLORAL SCENT PROFILES UNDER SELECTION PRESSURE SHOW MENDELIAN BASED GENETIC STRUCTURE AND EVOLVE VIA TRANSPOSON ACTIVITY

Victoria Ruiz-Hernández[1], Julia Weiss[1], Benjamin Hermans[1], Joëlle K. Mühlemann,[2,] Natalia Dudareva,[3] and Marcos Egea-Cortines[1]

[1] Genética Molecular, Instituto de Biotecnología Vegetal, Universidad Politécnica de Cartagena 30202 Cartagena, Spain, [2] *Department of Biology, Wake Forest University, North Carolina 27109, USA,* [3] *Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907, USA*

Scent and volatiles are an interface driving interaction between plants and a large array of organisms such as bacteria, fungi, hervibores or pollinators. Floral scent is formed by combinations of Volatile Organic Compounds. We have used the *Antirrhinum* genus as a model to study the genetic structure and evolution of scent as it comprises over 25 species with a history of intercrossing and evolution back to a parental type. Analysis of floral scent in eight *Antirrhinum* wild species and two *A. majus* lines identified 130 compounds such as phenylpropanoids, benzenoids, mono and sesquiterpenes, nitrogen containing compounds and aliphatic alcohols. Using the volatile profiles we were able to construct the phylogenetic subsections of the genus. Cluster analysis showed that scent is probably selected as a combination of components in most cases but single pathways may also be a target of selection. Despite the complexity of the scent profiles, a cross of *A. majus* x *A. linkianum* differing in methyl benzoate, methyl cinnamate, acetophenone and ocimene showed Mendelian segregations of these volatiles. The null *A. linkianum BENZOIC ACID CARBOXYMETHYL TRANSFERASE* showed multiple polymorphisms in the 5' promoter region including an *IDLE* MITE transposon insertion. The strong match between scent profiles and phylogeny, the complexity of blends based on combination of single genes coding for enzymes or regulatory elements coupled to transposon activity may allow rapid changes of scent profiles, which seem to be under strong evolutionary pressure.

Universidad Politécnica de Cartagena | GOBIERNO DE ESPAÑA | MINISTERIO DE ECONOMÍA Y COMPETITIVIDAD | MINISTERIO DE EDUCACIÓN, CULTURA Y DEPORTE | Genetics Technical University of Cartagena | Instituto de Biotecnología Vegetal, Universidad Politécnica de Cartagena

Complex floral scent profiles under selection pressure show Mendelian based genetic structure and evolve via transposon activity

Victoria Ruiz-Hernández[1], Julia Weiss[1], Benjamin Hermans[1], Joëlle K. Mühlemann,[2], Natalia Dudareva,[3] and Marcos Egea-Cortines[1]

[1] Genética Molecular, Instituto de Biotecnología Vegetal, Universidad Politécnica de Cartagena 30202 Cartagena, Spain, [2] *Department of Biology, Wake Forest University, North Carolina 27109, USA,* [3] *Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907, USA*

Background

- Scent and volatiles are an interface driving interaction between plants and a large array of organisms such as bacteria, fungi, herbivores or pollinators. Floral scent is formed by combinations of Volatile Organic Compounds (VOCs).
- We have used the *Antirrhinum* genus as a model to study the genetic structure and evolution of scent as it comprises over 25 species with a history of intercrossing and evolution back to a parental type.
- Analysis of floral scent in eight *Antirrhinum* wild species and two *A. majus* lines identified 130 compounds such as phenylpropanoids, benzenoids, mono- and sesquiterpenes, nitrogen-containing compounds and aliphatic alcohols.

Figure 1. Origin of seeds (filled circles) used in this study. For seeds of unknown origin, the area of distribution according to Vargas et al. (2009) is indicated (empty circle). *A. graniticum* is widely spread over central Iberian Penisula and the Western coast area, but absent in the North and East of the Iberian Peninsula (Vargas et al. 2010);

Phylogeny based on scent

Using the volatile profiles we were able to construct the phylogenetic subsections of the genus. Principal component analysis of the biosynthetic pathways of VOCs did not match with the phylogeny of the genus.

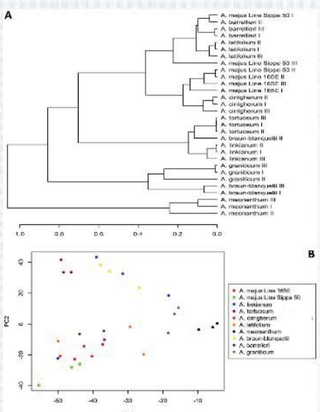

Figure 2. A) Phylogenetic tree based on VOCs profiles of the studied species; B) PCA based on scent biosynthetic pathways

Contrasting scent profiles

We crossed *A. majus* (165E) and *A. linkianum* because they have contrasting scent profiles.

Table 1. Percentaje of emission of major compounds in *A. majus* (165E) and *A. linkianum*. From flower opening until 6 days after

Volatile	*A. majus (165E)*	*A. linkianum*
Ocimene (Z)	15.10	42.30
Ocimene (E)	0.30	1.05
Acetophenone	32.57	0.07
Methyl Cinnamate	0.37	16.27
Methyl Benzoate	41.00	1.57

Mendelian segregations

We found F2 plants with high and low emission levels for the four main compounds. Segregation adjustment: methyl benzoate 3:1 (p-value= 0.581), methyl cinnamate 3:1 (p-value= 0.3218), acetophenone 9:7 (p-value=0,1966) and β-ocimene (Z) (p-value=0.1056).

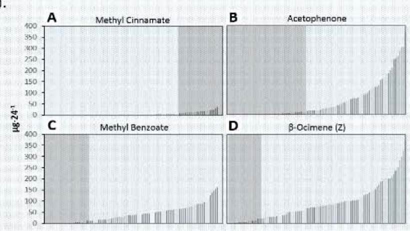

Figure 3. Darker areas indicate recessive alleles. Each bar indicates an independent F2 line

BAMT expression

We analysed the mRNA expression of *A. linkinaum BAMT* in F3 plants that segregated methyl benzoate production. Expression of the *BAMT* mRNA was undetectable in plants lacking methyl benzoate production suggesting that the *A. linkianum BAMT* was a null allele.

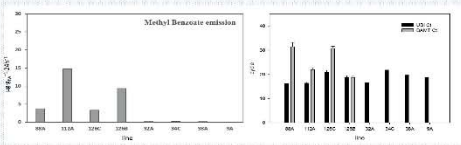

Figure 4. Methyl benzoate emission and *BAMT* expression

BAMT protein

We found a total of 40 SNPs in the coding region as compared to *A. linkianum*. These polymorphisms at the DNA level caused 14 aminoacid changes at the protein level.

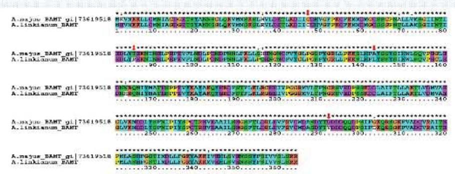

Figure 5. A. majus and A. linkianum BAMT protein alignment

BAMT promoter

We amplified and sequenced 2131 bp of the promoter region of *A. majus*, and compared it to the corresponding fragment of the *A. linkianum* genome. We found a set of rearrangements in *A. linkianum* promoter, including two major insertions with homologies with an Idle-MITE transposable element, a fragment found in the promoter region of the PLENA locus (BLASTN e-114) and the VENOSA genomic locus (BLASTN 2e-19).

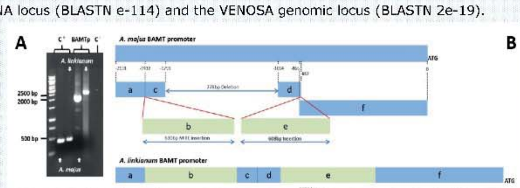

Figure 6. *BAMT* promoter in *A. majus* and *A. linkianum*

Conclusions

Scent profiles match with the phylogeny of the genus indicating a selection pressure or local adaptation of specific blends.

Altogether the complex rearrangement of the *A. linkianum* regulatory region creates a null allele.

Our data shows that genome evolution and traits under apparently strong evolutionary pressure may be subject to rapid changes as a result of transposon-mediated small-scale insertions and deletions.

XIII REUNIÓN DE BIOLOGÍA MOLECULAR DE PLANTAS

CARTAGENA, 11-13 Junio 2014

FLORAL VOLATILE PROFILE OF *ANTHIRRINUM* SPECIES AS A SPECIATION TRAIT REFLECTS THE PHYLOGENETIC STRUCTURE OF THE GENUS

Mª Victoria Ruíz-Hernández[1]; Benjamin Hermans[3]; María José Roca[2]; Julia Weiss[1]; and Marcos Egea-Cortines[1]

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The genus *Antirrhinum* comprises several species forming hybrids followed by phenotypic regression back to the original parental type. This evolutionary behaviour indicates the existence of a multilocus evolutionary pressure on dependent and/or independent characters. A comprehensive analysis of floral volatile organic compounds (VOCs) in nine *Antirrhinum* wild species and two *A. majus* lines identified a total of 130 VOCs. Of these compounds, 23 represented major compounds, belonging to the chemical categories: phenylpropanoids, benzenoids, mono and sesquiterpenes, nitrogen containing compounds and aliphatic alcohols. Each species had a different flower fragrance blend. Hierarchical cluster analysis of volatile profiles in the individual species confirmed the phylogenetic subsection assignation based on phenotypic markers. *A. meonanthum* and *A. braun blanquetii* of subsection *Streptosepalum* clearly diverged from subsection *Antirrhinum*. In accordance with phylogenetic analysis based on multilocus nuclear genotyping, *A. graniticum* did not cluster with the other species of subsection *Antirrhinum*, but with *A. braun blanquetii* from subsection *Streptosepalum*. Within subsection *Antirrhinum* we confirmed clustering of species within the 'majus complex'. We are constructing a recombinant inbred line of A. majus x A. linkianum and we found in the F2 independent plants that did not produce acetophenone, ocimene, methyl benzoate or methyl cinnamate. Despite the complexity of the scent profiles, scent production followed a mendelian segregation. We found that plants without methyl benzoate did not produce *BENZOIC ACID METHYL TRANSFERASE* (*BAMT*) mRNA, suggesting that the identified differences in scent profiles could be the result of combinations of mutations in a single loci coding for scent synthesis enzymes, rather than regulatory loci that might have a larger impact in the complete scent profile.

Floral volatile profile of *Anthirrinum* species as a speciation trait which reflects the phylogenetic structure of the genus

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Introduction

•The genus *Antirrhinum* comprises approx. 25 species belonging to three morphological subsections (Rothmaler 1956; Webb 1971; Sutton 1988). Species may form hybrids followed by phenotypic regression back to the original parental type, indicating multilocus evolutionary pressure (Khimoun et al. 2011; Wilson and Hudson 2011).

•Certain floral scent preferences might be responsible for preferential pollination of hybrids with parental type scent profile, leading to the regression phenomenon. This would implicate some degree of consistency between the molecular data supporting the snapdragon clades and scent profiles of the different species.

•Two species with a strongly diverging volatile organic compound (VOC) profile, *A. majus* x *A. linkianum* , were selected for construction of a recombinant inbred line.

FIG. 1. (A) A. majus line 165E (B) A. majus line Sippe 50 (C) A. majus subsp. linkianum (D) A. majus subsp. tortuosum (E) A. majus subsp. cirrigherum (F) A. latifolium (G) A. meonanthum (H) A. braun-blanquetii (I) A. barrelieri (J) A. graniticum

Aim

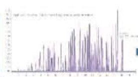
•Determine floral VOC profiles of ten *Anthirrhinum* species (Fig. 1) for cluster analysis and comparison with molecular data.

•Follow segregation of individual VOCs in the F2 population of a cross between *A. majus* x *A. linkianum*

•Analyze plants lacking methyl benzoate, for production of the corresponding mRNA, *BENZOIC ACID METHYL TRANSFERASE* (*BAMT*) *(Negre et al. 2003)* in order to investigate the importance of mutations in single loci coding for scent synthesis.

Methodology

Floral VOC collection by headspace analysis over a 24 hour period and *Gas-chromatography mass spectrometry*



Hierarchical cluster analysis of species and developmental stages

Construction of a RIL population from *A. majus x A. linkianum*

Segregation analysis of F2 plants for individual VOCs

Analysis of *BENZOIC ACID METHYL TRANSFERASE* (*BAMT*) *expression*

qPCR for BAMT in plants of F2-RIL population, producing and lacking methyl benzoate

Results

○ Each species has a unique scent profile (Table 2 exemplarily for *A. majus* and *A. linkianum*) which cluster in a way that reflects their likely evolutionary relationships found by DNA analysis (Fig. 2)

Table 2. Percentage of major volatile compounds *A. majus* and *A. linkianum*

A)	*A. majus* line 165E			*A. majus ssp. linkianum*		
Flower color	pink-white			magenta		
Flower opening stage	I	II	III	I	II	III
Benzaldehyde	3.0	2.8	1.3	3.1	0.5	
Acetophenone	20.3	33.5	43.9		0.2	
1-(2-hydroxyphenyl) ethanone	0.6	0.7	0.9			
Methyl benzoate	55.7	41.7	25.6	1.5	3.0	0.2
3.5-Dimethoxytoluene	0.9	0.2	0.3			
β-Myrcene	2.8	2.7	6.0	5.4	4.3	4.9
β-Ocimene (Z)	12.5	14.5	18.3	23.5	44.1	59.3
β-Ocimene (E)		0.3			0.9	1.2
Linalool					0.3	0.7
α-Farnesene	0.3	0.1	0.1	1.8	2.4	4
Methyl cinnamate	0.5	0.2	0.4	13.7	22.7	12.4
Cinnamyl alcohol	0.3		0.5	1.5	2.6	0.3
2-Ethyl 1-Hexanol		0.5	0.9	20.5	3.8	3.6

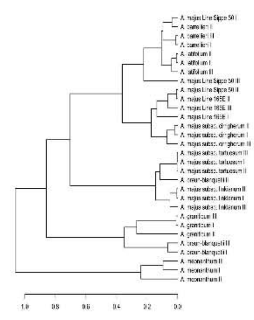


Fig. 2 Hierarchical cluster analysis of *Antirrhinum* species based on the floral fragrance matrix.

○ Independent plants of the RIL-F2 population did not produce acetophenone, ocimene, methyl benzoate or methyl cinnamate. Despite the complexity of the scent profiles, scent production followed a mendelian segregation (shown for methyl benzoate – Table 3)

Table 3. Distribution of methyl benzoate in terms of peak area in the chromatogram and χ^2-test for segregation of methyl benzoate

Groups	Range	Plant Number
P	$6.4\cdot10^5$	1
Q	$1.2\cdot10^7 - 8.2\cdot10^7$	23
R	$1.1\cdot10^8 - 9.9\cdot10^8$	70
S	$1.0\cdot10^9 - 2.4\cdot10^9$	16

Recessive Group	Dominant Group	Segregation	p value
P+Q	R+S	1:3	0.4409

F3 plants show a mixture of fixed alleles as homozygotes and a possible reversion of plants that do not produce acetophenone back to wild type

Name	Previous phenotype		Descendants ratio low/high	Mendelian meaning
126	Ocimene	high	1/3	Heterozygote segregating
	Acetophenone	high	4	Homozygote fixed
	Methyl benzoate	high	2/2	Heterozygote segregating
	Methyl cinnamate	high	4	Homozygote fixed
9	Acetophenone	low	0/7	Possible revertant
	Methyl benzoate	low	7/0	Homozygote fixed
86	Methyl cinnamate	low	2/2	Heterozygote segregating
13	Acetophenone	high	0/4	Homozygote fixed
	Methyl cinnamate	high	0/4	Homozygote fixed

Conclusion

•Selection involving scent profiles has been important in diversification of *Antirrhinum* species and might contribute to their speciation.

•Despite the complexity of the scent profiles, scent production followed a mendelian segregation,.

•Plants without methyl benzoate did not produce *BENZOIC ACID METHYL TRANSFERASE* (*BAMT*) mRNA suggesting that the identified differences in scent profiles could be the result of combinations of mutations in single loci coding for scent synthesis enzymes, rather than regulatory loci that might have a larger impact in the complete scent profile.

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Oral communications presented by Victoria Ruiz-Hernández

Understanding genetic mechanisms underpinning volatile emission. Victoria Ruiz-Hernández, Julia Weiss and Marcos Egea Cortines.

Organizer: Programa de Doctorado en Técnicas Avanzadas de Investigación y Desarrollo Agrario y Alimentario

Congress: 7th Workshop on agri food research

Location: Cartagena, Spain

Date: 7 8/5/2018

The importance of aromas in plants. Victoria Ruiz-Hernández, Julia Weiss, Pablo Bielza, Beverley Glover, Marcos Egea Cortines.

Organizer: Escuela Internacional de Doctorado (EINDOC UPCT)

Desarrollo Agrario y Alimentario

Congress: I JORNADAS DOCTORALES UPCT: DOCTORADO EMPRESA

Location: Cartagena, Spain

Date: 7 8/5/2018

Circadian emission of main volatile compounds in *Antirrhinum majus*. Victoria Ruiz-Hernández, Julia Weiss and Marcos Egea Cortines.

Organizer: Programa de Doctorado en Técnicas Avanzadas de Investigación y Desarrollo Agrario y Alimentario

Congress: 5th Workshop on agri food research

Location: Cartagena, Spain

Date: 9 10/5/2016

ISBN: 978 84 16325 29 0

Identification of DNA from *Antirrhinum linkianum* introgressed in *A. majus* with common molecular markers. Victoria Ruiz-Hernández, Julia Weiss and Marcos Egea Cortines.

Organizer: Programa de Doctorado en Técnicas Avanzadas de Investigación y Desarrollo Agrario y Alimentario

Congress: 4th Workshop on agri food research

Location: Cartagena, Spain

Date: 11 12/5/2015

ISBN: 978 84 608 5399 2

Desarrollo de marcadores moleculares para *Antirrhinum linkianum* basados en el mapa genético de *Antirrhinum majus*. Victoria Ruiz-Hernández, Julia Weiss and Marcos Egea Cortines.

Organizer: Asociación de Jóvenes Investigadores de Cartagena (AJICT)

Congress: VIII jornadas de Introducción a la Investigación

Location: Cartagena, Spain

Date: 28/5/2015

Oral communications presented with contributions of Victoria Ruiz-Hernández

Genetic and environmental factors affecting scent and volatiles production. Marcos Egea Cortines, Julia Weiss, **Victoria Ruiz-Hernández** and Marta Terry López.

Organizer: BIT Group Global Ltd.

Participation: Comunicación invitada

Congress: BIT's 6th Annual World Gene Convention 2015

Location: Shangri La Hotel (SLH), Qingdao, China

Date: 13 15/11/2015

Speaker: Marcos Egea Cortines

Interplay of continuous and circadian processes during flower development. Julia Weiss, Marta Terry López, Fernándo Pérez Sanz, **Victoria Ruíz-Hernández**, Pedro J. Navarro, Joëlle Muhlemann, Natalia Dudareva and Marcos Egea Cortines

Organizer: IPMB 2015

Participation: Comunicación oral

Congress: 11th International Congress of Plant Molecular Biology

Location: Iguazú Falls, Argentina Brazil

Date: 25 30/10/2015

Speaker: Marcos Egea Cortines

Communications unrelated to the PhD thesis

Effect of silencing *PhZTL* on growth and scent emission in *Petunia*. Marta Isabel Terry, Fernando Pérez Sanz, María Victoria Díaz Galián, **Victoria Ruiz-Hernández**, Pedro J. Navarro , Marcos Egea Cortines, Julia Weiss.

Participation: Poster

Congress: Xiv Reunión de Biología Molecular de Plantas

Location: Salamanca, Spain

Date: 4 6/7/2018

Caracterización de variedades autóctonas de caupí (*Vigna unguiculata* (L.) Walp). Marina Martos Fuentes, **Victoria Ruiz-Hernández**, Julia Weiss, Catalina Egea Gilabert, Juan A. Fernández, Marcos Egea Cortines.

Organizer: Ministerio de Agricultura, Alimentación y Medio Ambiente

Participation: Poster

Congress: XLV Seminario de Técnicos y Especialistas en Horticultura

Location: Zaragoza, Spain

Date: 15 19/6/2015

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Genetic and growth conditions determine the protein content in cowpea (*Vigna unguiculata*). Marina Martos Fuentes; Virginia Sánchez Navarro; **Victoria Ruiz-Hernández**; Julia Weiss; Catalina Egea Gilabert; Raúl Zornoza; Ángel Faz; Juan A. Fernández; Marcos Egea Cortines.

Organizer: The Protein Crops Working Group of the European Association for research on Plant Breeding (EUCARPIA), Oil and Protein Crops Section (OPC), and the Misión Biológica de Galicia (MBG) of the Spanish Nat.

Participation: Poster

Congress: Eucarpia International Symposium on protein crops (V Meeting AEL)

Location: Pontevedra, Spain

Date: 4 7/5/2015

Identification of clock genes involved in gating of volatile emission in Petunia. Julia Weiss; Michiel Vandennbussche; Miguel Ángel Casanova; **Victoria Ruiz-Hernández**; Marcos Egea Cortines.

Organizer: Université de Fribourg

Participation: Oral

Congress: 14th World Petunia Days

Location: Fribourg, Switzerland

Date: 9 12/4/2015

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Agradecimientos - Acknowledgements

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